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(54) Title: AN AUTOREGULATORY TETRACYCLINE-REGULATED SYSTEM FOR INDUCIBLE GENE EXPRESSION IN EUCARYOTES

(57) Abstract

A tetracycline-regulated system which provides autoregulatory, inducible gene expression in cultured cells and transgenic animals is described. In the autoregulatory plasmid pTet-tTAk, a modified tTA gene called tTAk was placed under the control of Tetp. Tetracycline prevents tTA from binding to Tetp, preventing expression of both tTA and luciferase. This negative feedback cycle ensures that little or no tTA is produced in the presence of tetracycline, thereby reducing or eliminating possible toxic effects. When tetracycline is removed, however, this strategy predicts that tiny amounts of tTA protein (which may result from the leakiness of the minimal promoter), will bind to Tet-op and stimulate expression of the tTAk gene. A positive feedforward loop is initiated which in turn leads to higher levels of expression of tTA and thus, luciferase. Polynucleotide molecules encoding the autoregulatory system, as well as methods of enhancing or decreasing the expression of desired genes, and kits for carrying out these methods are described.

An Autoregulatory Tetracycline-Regulated System For Inducible Gene Expression In Eucaryotes

Background of the Invention

Statement as to Rights to Inventions Made Under Federally-Sponsored Research and Development

Part of the work performed during development of this invention utilized U.S. Government funds. The U.S. Government has certain rights in this invention.

Field of the Invention

The invention is related to the recombinant DNA technology. A tetracycline-regulated system which provides autoregulatory, inducible gene expression in cultured cells and transgenic animals is described.

Related Art

Systems for inducible mammalian gene expression have typically encountered limitations such as basal leakiness, toxic or nonspecific effects of inducing agents or treatments, limited cell type applicability and low levels of expression (reviewed in Yarranton, G. T., *Curr. Opin. Biotech. 3*:506-511 (1992)). Recently, a system was described (Gossen, M. & Bujard, H., *Proc. Natl. Acad. Sci. USA 89*:5547-5551 (1992)) that overcomes many of these difficulties by placing target genes under the control of a regulatory sequence (*tet*O) from the tetracycline-resistance operon of Tn10. In bacteria, this short sequence is bound tightly by the tetracycline repressor protein (*tet*R), and binding is blocked by the antibiotic tetracycline (Hillen, W. & Wissmann, A., in *Protein-Nucleic Acid Interaction, Topics in Molecular and Structural Biology*, Saenger, W. &

Heinemann, U., eds., Macmillan, London (1989), pp. 143-162). A hybrid fusion protein, the tetracycline transactivator (tTA), combines the *tet*R DNA binding domain with the transcriptional activation domain of VP-16, such that when tTA binds to a minimal promoter containing *tet*O sequences, transcription of the target gene is activated. Tetracycline binding to tTA prevents activation presumably by causing a conformational change in the *tetR* portion of tTA which blocks binding of tTA to *tet*O (Hinrichs, W., *et al.*, *Science 264*:418-420 (1994)); gene activation is achieved by removing tetracycline (Gossen, M. & Bujard, H., *Proc. Natl. Acad. Sci. USA 89*:5547-5551 (1992)).

The primary limitation of this system is difficulty in expressing even moderate levels of the tTA protein (undetectable by western blotting and barely detectable by gel electrophoresis mobility shift assay (Gossen, M. & Bujard, H., *Proc. Natl. Acad. Sci. USA 89*:5547-5551 (1992))). Gossen and Bujard speculated that this was due to transcriptional "squelching" (Gill, G. & Ptashne, M., *Nature (London) 334*:721-724 (1988)) by the VP16 transactivator domain leading to death of cells expressing even modest levels of the tTA protein. These results combined with the observation of an apparently low level of expression of an inducible luciferase transgene using this system (Furth, P. A., *et al.*, *Proc. Natl. Acad. Sci. USA 91*:9302-9306 (1994)) suggest that inefficiencies in tTA expression may contribute to the difficulty.

Summary of the Invention

By placing the tTA gene under the control of a promoter containing *tet*O, an autoregulatory tTA expression vector is created that allows high levels of tTA expression. It is demonstrated herein that this strategy permits the creation of highly inducible transfected cells with much greater efficiency than the constitutive system. Furthermore, it allows the creation of transgenic mice in which expression of a luciferase reporter gene can be controlled by altering the concentration of tetracycline in the drinking water of the animals. The

autoregulated expression of transactivator protein should make the tetracycline system applicable to a wide array of problems requiring inducible mammalian gene expression.

The first embodiment of the invention relates to a composition of matter comprising a polynucleotide molecule encoding a tetracycline transactivator fusion protein, said protein comprising a prokaryotic *tet* repressor and a eucaryotic transcriptional activator protein, and said polynucleotide molecule being operably linked to an inducible minimal promoter, which promoter contains at least one *tet* operator sequence. The open reading frame of the polynucleotide molecule encoding the tetracycline transactivator fusion protein is modified at its 5′ end to provide an optimal context for translational initiation. In a preferred embodiment of the invention, it is modified to provide a unique restriction site, such as *Hind*III. In the most preferred embodiment of this invention, the open reading frame of the polynucleotide molecule encoding the tetracycline transactivator fusion protein is modified at its 5′ end to encode an oligonucleotide identified as SEQ ID NO. ——. In a preferred embodiment, the polynucleotide molecule encoding a tetracycline transactivator fusion protein is DNA.

The second embodiment of the invention relates to a cloning vector containing the polynucleotide molecule of the invention. The most preferred embodiments of the invention relate to plasmids pTet-Splice and pTet-tTAK.

The third embodiment of the invention relates to a eucaryotic cell transfected with the polynucleotide molecule of the present invention. In a preferred embodiment, the eucaryotic cell contains tetracycline in an amount sufficient to suppress binding of tetracycline transactivator fusion protein to said inducible minimal promoter. In another preferred embodiment of the invention, the eucaryotic cell is further transfected with a polynucleotide molecule encoding a heterologous protein operably linked to an inducible minimal promoter, which contains at least one *tet* operator sequence. In the most preferred embodiment of the invention, at least one of the polynucleotide molecules is operably linked to a minimal promoter and seven *tet* operator sequences. In a further preferred

embodiment, the polynucleotide molecule encoding a tetracycline transactivator fusion protein is expressed in an amount sufficient to drive expression of the polynucleotide molecule, encoding the heterologous protein, in the absence of tetracycline. In another preferred embodiment, the tetracycline transactivator fusion protein is present in an amount sufficient to drive expression of the heterologous protein.

The fourth embodiment of the invention relates to a method to decrease or shut off expression of a heterologous protein comprising

(a) transforming a eucaryotic cell with

(i) a first polynucleotide molecule encoding a tetracyline transactivator fusion protein, said protein comprising a prokaryotic *tet* repressor and a eucaryotic transcriptional activator protein, and said polynucleotide molecule being operably linked to an inducible minimal promoter, which promoter contains at least one *tet* operator sequence;

(ii) a second polynucleotide molecule encoding the heterologous protein, said protein being operably linked to an inducible minimal promoter, and said promoter containing at least one *tet* operator sequence; and

(b) cultivating the eucaryotic cell in a medium comprising tetracycline or a tetracycline analogue. In a preferred embodiment, the second polynucleotide molecule is operably linked to a minimal promoter and seven *tet* operator sequences.

The fifth embodiment of the invention relates to a method to activate or enhance the expression of a heterologous protein comprising

(a) transforming a eucaryotic cell with

(i) a first polynucleotide molecule encoding tetracycline transactivator fusion protein, said protein comprising a prokaryotic *tet* repressor and a eucaryotic transcriptional activator protein, and said polynucleotide molecule being operably linked to an inducible promoter, which promoter contains at least one *tet* operator sequence;

(ii) a second polynucleotide molecule encoding the heterologous protein, said protein being operably linked to an inducible minimal promoter, and said promoter containing at least one *tet* operator sequence; and

(b) cultivating the eucaryotic cell in a medium lacking tetracycline or a tetracycline analogue.

The sixth embodiment of the invention relates to a kit comprising a carrier means having in close confinement therein at least two container means, wherein a first container means contains a first polynucleotide molecule encoding a tetracycline transactivator fusion protein, said protein comprising a procaryotic *tet* repressor and a eucaryotic transcriptional activator protein, and said polynucleotide molecule being operably linked to an inducible minimal promoter, which promoter contains at least one *tet* operator sequence; and a second container means contains a second polynucleotide molecule encoding said inducible minimal promoter, which promoter contains at least one *tet* operator sequence, which *tet* operator sequence is strategically positioned for being operably linked to a heterologous polynucleotide sequence encoding a polypeptide.

The seventh embodiment of the invention relates to a kit comprising a carrier means having in close confinement therein at least two container means, wherein a first container means contains a eucaryotic cell transfected with a first polynucleotide molecule encoding a tetracycline transactivator fusion protein, said protein comprising a procaryotic *tet* repressor and a eucaryotic transcriptional activator protein, and said polynucleotide molecule being operably linked to an inducible minimal promoter, which promoter contains at least one *tet* operator sequence; and a second container means contains a second polynucleotide molecule comprising an inducible minimal promoter, which promoter contains at least one *tet* operator sequence, which *tet* operator sequence is strategically positioned for being operably linked to a heterologous polynucleotide sequence encoding a heterologous polypeptide.

The polynucleotides described in this invention and cell lines containing said polynucleotides are research tools which allow one to tightly and quantitatively control the expression of a large variety of genes. This is of interest in broad areas of basic as well as applied research.

The invention also relates to the construction of eucaryotic production cell lines and strains in which the synthesis of the product. RNA or protein, is controlled by the *tet* regulatory system. These cell lines and strains allow one to induce protein synthesis at a predetermined time point or within a time window during a fermentation process. This control allows one to synthesize in large scale cultures gene products whose prolonged presence is lethal to the cells. Alternatively, the cells allow one to induce production of RNA when it is desirable to generate RNA molecules used to achieve a variety of cellular tasks, regulation, and function. Induction of RNA production can be controlled where, for example, the RNA are used as antisense oligos to inhibit the function of a gene which is either homologous or heterologous to the cell.

The invention also relates to the construction of cell lines which can be used in screening systems to identify compounds of pharmaceutical or other commercial value. In such systems, the expression of target molecules including but not limited to receptors such as the GABA or estrogen receptor, whose long term presence, in particular, in high copy numbers is often cell damaging, can be temporarily and quantitatively controlled.

The invention also relates to the construction of transgenic animals in which the expression of a single gene can be controlled externally by the *tet* regulatory system. Such genes include human genes whose expression, failure of expression, or other defects are involved in human diseases. Such transgenic animals can serve as models for human diseases in therapeutic studies and for the screening of compounds of pharmaceutical interest. The invention also relates to the construction of transgenic animals for the production of compounds of pharmaceutical or other commercial interest.

Another important application of this system makes possible the temporal control of gene expression, where for example, the gene of interest is introduced into a cell, animal, or plant to compensate for lethal knock out of certain genes in the transgene. Using the system to introduce a copy of the gene which has been suppressed or deactivated, enough protein or RNA can be produced to allow growth and development of the cell or the plant or animal until a time at which it is desired to shut off production of the gene and carry out the manipulations that require the lethal knock out of said gene.

Brief Description of the Figures

Figure 1 is a schematic representation depicting the autoregulatory strategy for inducible gene expression. Autoregulatory expression of tTA is accomplished in pTet-tTAk by placing the tTAk gene (white box) under the control of Tetp consisting of seven copies of the tetracycline operator sequence (Tet-op; dark shaded box) upstream of the minimal human cytomegalovirus (hCMV) promoter region containing a TATA box and transcription start site (black circle). The luciferase reporter gene (shaded box) of pUHC13-3 is also controlled by the Tetp promoter. The tTA protein is shown as two adjoining striped boxes to represent the two domains of the protein (for DNA binding and transactivation). In the presence of tetracycline (left panel), the basal activity of the minimal hCMV promoter results in expression of very low levels of the tTA protein (represented as a small tTA icon), and any tTA protein produced is blocked from binding to Tet-op. Both luciferase and tTA expression are therefore maintained at low levels (thin, short dashed lines). When tetracycline is removed (right panel), the small amounts of tTA present bind Tet-op, stimulating expression of the tTA gene. Higher levels of the tTA protein now stimulate higher levels of tTA and thus, luciferase expression (heavy, long dashed lines).

Figures 2A and 2B are bar graphs which depict inducible V(D)J recombination in NIH3T3 fibroblasts.

Figure 2A depicts a bar graph analysis of clones containing pcDNA-tTAk (constitutive tTA expression). Seventeen stable transfectant clones (S1-1 to S1-17) were derived and assayed for the ability to carry out V(D)J recombination by transient co-transfection with a V(D)J recombination substrate and Tetp-controlled RAG-1 and RAG-2 expression vectors. Parallel transfections were performed in the presence (Tet+) and absence (Tet-) of tetracycline in the growth media, and the V(D)J recombination frequency (expressed as a percent) was determined as described in Materials and Methods. For comparison, four control assays performed in NIH3T3 cells are also shown (first two samples): transfection of the recombination substrate in the absence of RAG-1 and RAG-2, with and without tetracycline, and co-transfection of the recombination substrate with constitutive RAG expression vectors, with and without tetracycline. Tetracycline had no effect on V(D)J recombination frequency when RAG-1 and RAG-2 were expressed from constitutive hCMV promoters. Fold induction achieved by removing tetracycline is indicated above the bars in cases where clearly detectable recombination was observed.

Figure 2B depicts a bar graph analysis of clones containing pTet-tTAk (autoregulatory tTA expression). Ten stable transfectant clones (S2-1 to S2-10) and two clones containing pTet-tTAk, pTet-R1A/C and pTet-R2A (S4-9 and S4-5) were assayed for the ability to carry out V(D)J recombination as described above. The first two samples are the same control samples described in Fig. 2A. Note the difference in the recombination frequency axis scale between Fig. 2A and Fig. 2B. Asterisks (*) mark two Tet+ transfections that yielded very small numbers of ampicillin resistant colonies, making the calculated recombination frequency unreliable. Consequently, the fold-inducibility for these clones is not shown. The number of ampicillin resistant colonies was low in these experiments (range, 350-55,550). Based on additional assays on some of the cell lines, we estimate that the reported recombination frequencies are as much as two fold overestimates in both Fig. 2A and Fig. 2B.

Figures 3A, 3B, and 3C are photographs of RNA blots and a Western blot depicting the detection of mRNA and protein expression activated using the inducible, autoregulatory system.

Figure 3A is a photograph of a RNA blot of total cell RNA from S4-9 (stable co-transfectant of pTet-tTAk, pTet-R1A/C, and pTet-R2A) cultured 23h in the presence or absence of tetracycline, and S2-6 (stable transfectant of pTet-tTAk) transiently transfected with either pTet-R1 or pTet-R2 and cultured for 48 hours in the presence or absence of tetracycline. Blots were sequentially hybridized with probes detecting tTAk, RAG-1 and/or RAG-2, and γ-actin mRNA.

Figure 3B is a photograph of a Western blot of cell extracts from S2-6 cells cultured for 48 hours in the presence or absence of tetracycline. Blot was probed with anti-*tet* R antibody-containing hybridoma supernatant which detects the tTA protein. The dye front is indicated.

Figure 3C is a photograph of a blot of total cell RNA from thymus (T) and lung (L) of pTet-tTAk/Tet-luciferase transgenic mice maintained for 7 days in the presence or absence of tetracycline in their drinking water. Approximately 20 μg of RNA was loaded per lane.

Figure 4 is a graph depicting inducible luciferase activity in tissues of transgenic mice. Values represent the relative light units (rLU) (with lysis buffer background subtracted) per mg protein in tissue lysates from 4-7 week old mice maintained for 7-8 days in the presence or absence of tetracycline in their drinking water. Open triangles are transgene negative mice; open circles are uninduced transgene positive mice; and closed circles are induced transgene positive mice. Mice were genetically identical with respect to the transgenes. Results are compiled from three separate experiments.

Figure 5 is a graph depicting the ability of pTet-tTAk to induce expression of luciferase activity in a transfected fibroblast cell line. pUHC13-3 was co-transfected with either pTet-tTAk or pcDNA-tTAk into NIH3T3 fibroblast cells, and 48 hours later the cells were harvested and the luciferase light

units present in the extracts was measured. pTet-tTAk transfections were performed in the presence and absence of tetracycline (tet), while pcDNA-tTAk transfection was performed only in the absence of tet. It is important to note that the luciferase values have not been corrected for transfection efficiency. In addition, under the conditions used, pcDNA-tTAk would replicate inside the cells while pTet-tTAk would not. Therefore, comparisons between the values obtained with pTet-tTAk and pcDNA-tTAk are not meaningful.

Figure 6 is a photograph of an autoradiograph depicting loss of tTA protein at 16 days without tetracycline in S2-6 cells. Figure 6 is a photograph of a Western blot of cell extracts from cultured S2-6 and S2-1 cell lines. First lane: marker proteins (no bands, visible); second and third lanes: S2-6 cells grown in the absence of tet for 16 days; fourth and fifth lanes: S2-6 cells grown in the presence of 0.5 μg/ml tet for 16 days; sixth lane: S2-1 cells grown in the presence of 0.5 μg/ml tet for 16 days; and seventh lane: S2-6 cells grown in the absence of tet for 2 days. A signal for the tTA protein is seen in S2-6 cells grown in the absence of tet for 2 days, but not in the same cells cultured in the absence of tet for 16 days. The band seen at the bottom in lanes 2-7 ("protein front") is the dye front and represents a non-specific signal.

Figure 7 is a schematic depiction of the construction of pCMV-tTAk, pcDNAI-neo, and pcDNA-tTAk.

Figure 8 is a schematic depiction of the construction of pTet-Splice and pTet-tTAk.

Figure 9A depicts a restriction map of pTet-Splice. Cloning sites are shown in boldface print. Note that there are two *Eco*RI sites.

Figures 9B-9G depict the nucleotide sequence of pTet-Splice (SEQ ID NO ---).

Figure 10A depicts a restriction map of pTet-tTAk.

Figures 10B-10G depict the nucleotide as well as partial amino acid sequence of pTet-tTAk (SEQ ID NO ---).

Figure 11A depicts a restriction map of pUHD15-1.

Figures 11B-11F depict the nucleotide as well as partial amino acid sequence of pUHD15-1.

Figure 12A depicts a restriction map of pUHC-13-3.

Figures 12B-12F depict the nucleotide as well as partial amino acid sequence of pUHC-13-3 (SEQ ID NO ---).

Detailed Description of the Preferred Embodiments

A system for tetracycline-regulated inducible gene expression was described recently which relies on constitutive expression of a transactivator fusion protein (tTA) consisting of the DNA binding domain of the tetracycline repressor and the transcriptional activation domain of VP16 (U.S. patent application serial number 08/076,726, herein incorporated by reference in its entirety; Gossen, M. & Bujard, H., *Proc. Natl. Acad. Sci. USA 89*:5547-5551 (1992)). This system yielded only low levels of transactivator protein, probably because tTA is toxic. To avoid this difficulty, the tTA gene was placed under the control of the inducible promoter to which tTA binds, making expression of tTA itself inducible and autoregulatory.

When used to drive expression of the recombination activating genes RAG-1 and RAG-2, the autoregulatory system yielded both substantially higher levels of V(D)J recombination activity (70 fold on average) and inducible expression in a much larger fraction of transfected cells (autoregulatory, 90% vs. constitutive, 18%). In addition, this system allowed the creation of inducible transgenic mice in which expression of a luciferase transgene was induced tens to hundreds fold the basal levels in most tissues examined. Induced levels of expression were highest in thymus and lung and appear to be substantially higher than in previously reported inducible luciferase transgenic mice created with the constitutive system. With the modified system, inducible transactivator mRNA and protein were easily detected in cell lines by RNA and western blotting, and transactivator mRNA was detected by RNA blotting in some tissues of transgenic

mice. This autoregulatory system represents an improved strategy for tetracycline regulated gene expression both in cultured cells and in transgenic animals.

As mentioned above, the inducible tetracycline expression system described recently (Gossen, M. & Bujard, H., *Proc. Natl. Acad. Sci. USA 89*:5547-5551 (1992)) relies on constitutive expression of the tTA gene from a fully functional human cytomegalovirus (hCMV) promoter, and a luciferase reporter gene under the control of the inducible promoter Tetp. In this system, tetracycline prevents the activation of luciferase gene expression, but does not prevent the tTA protein from exerting potentially deleterious effects on cells (Gill, G. & Ptashne, M., *Nature (London) 334*:721-724 (1988)).

Hence, in the autoregulatory plasmid pTet-tTAk, a modified tTA gene called tTAk was placed under the control of Tetp (Figure 1). Tetracycline prevents tTA from binding to Tetp, preventing expression of both tTA and luciferase. This negative feedback cycle ensures that little or no tTA is produced in the presence of tetracycline, thereby reducing or eliminating possible toxic effects. When tetracycline is removed, however, this strategy predicts that tiny amounts of tTA protein (which may result from the leakiness of the minimal promoter), will bind to Tet-op and stimulate expression of the tTAk gene. A positive feedforward loop is initiated which in turn leads to higher levels of expression of tTA and thus, luciferase (Figure 1). For constitutive expression of tTA, the tTAk gene was placed under the control of the hCMV promoter, followed with additional sequences to direct RNA splicing and polyadenylation of the tTA transcript. This plasmid (pcDNA-tTAk) also includes the neo gene, which allows for selection of the plasmid in mammalian cells.

The present invention relates to an autoregulatory control system that in eucaryotic cells allows regulation of expression of an individual gene over 200 to 3700 fold. This system is based on regulatory elements of a tetracycline-resistance operon, e.g. Tn*10* of *E. coli* (Hillen & Wissmann, "Topics in Molecular and Structural Biology," in *Protein-Nucleic Acid Interaction*, Saeger & Heinemann, eds., Macmillan, London, 1989, Vol. 10, pp. 143-162), in which

transcription of resistance-mediating genes is negatively regulated by a tetracycline repressor (*tet*R). In the presence of tetracycline or a tetracycline analogue, *tet*R does not bind to its operators located within the promoter region of the operon and allows transcription. By combining *tet*R with a protein domain capable of activating transcription in eucaryotes, such as (i) acidic domains (e.g. the C-terminal domain of VP16 from HSV (Triezenberg *et al.*, *Genes Dev. 2*:718-729 (1988)) or empirically determined, non-eucaryotic acidic domains identified by genetic means (Giniger and Ptashne, *Nature 330*:670-672 (1987))) or (ii) proline rich domains (e.g. that of CTF/NF-1 (Mermod *et al.*, *Cell 58*:741-753 (1989))) or (iii) serine/threonine rich domains (e.g. that of Oct-2 (Tanaka and Herr, *Cell 60*:375-386 (1990))) or (iv) glutamine rich domains (e.g. that of Sp1 (Courey and Tjian, *Cell 55*:867-898 (1988))) a hybrid transactivator is generated that stimulates minimal promoters fused to tetracycline operator (*tet*O) sequences. These promoters are virtually silent in the presence of low concentrations of tetracycline, which prevents the tetracycline-controlled transactivator (tTA) from binding to *tet*O sequences.

The specificity of the *tet*R for its operator sequence (Hillen & Wissmann, "Topics in Molecular and Structural Biology," in *Protein-Nucleic Acid Interaction*, Saeger & Heinemann, eds., Macmillan, London, 1989, Vol. 10, pp. 143-162) as well as the high affinity of tetracycline for *tet*R (Takahashi *et al.*, *J. Mol. Biol. 187*:341-348 (1986)) and the well-studied chemical and physiological properties of tetracyclines constitute a basis for an autoregulatory inducible expression system in eucaryotic cells far superior to the *lac*R/O/IPTG system.

In particular, the invention relates to a first polynucleotide molecule coding for a transactivator fusion protein comprising the *tet* repressor (*tet*R) and a protein domain capable of activating transcription in eucaryotes, wherein the first polynucleotide molecule is operably linked to an inducible minimal promoter, which promoter contains at least one *tet* operator sequence. The polynucleotide coding for *tet*R may be obtained according to Postle *et al.*, *Nucl.*

Acids Res. 12:4849-4863 (1984), the contents of which are fully incorporated by reference herein. Other *tet*R sequences and the respective binding sites for these repressors are identified (Waters *et al.*, *Nucl. Acids Res. 11*:6089-6105 (1983); Postle *et al.*, *Nucl. Acids Res. 12*:4849-4863 (1984); Unger *et al.*, *Gene 31*:103-108 (1984); Unger *et al.*, *Nucl. Acids Res. 12*:7693-7703 (1984); Tovar *et al.*, *Mol. Gen. Genet. 215*:76-80 (1988); for comparison and overview see Hillen and Wissmann in *Protein-Nucleic Acid Interaction*, Topics in Molecular and Structural Biology, Saenger and Heinemann (eds.), Macmillan, London, Vol. 10, pp. 143-162 (1989)) and can also be utilized for the expression system described.

The polynucleotide coding for the negatively charged C-terminal domain of HSV-16, a protein known to be a powerful transcription transactivator in eucaryotes, may be obtained according to Triezenberg *et al.*, *Genes Dev. 2*:718-729 (1988), the contents of which are fully incorporated by reference herein. Preferably, the activating domain comprises the C-terminal 130 amino acids of the virion protein 16.

The polynucleotide molecule coding for *tet*R may be linked to a polynucleotide molecule coding for the activating domain of HSV-16 and recombined with vector DNA in accordance with conventional techniques, including blunt-ended or stagger-ended termini for ligation, restriction enzyme digestion to provide appropriate termini, filling in of cohesive ends as appropriate, alkaline phosphatase treatment to avoid undesirable joining, and ligation with appropriate ligases.

The *tet*O sequence may be obtained, for example, according to Hillen & Wissmann, "Topics in Molecular and Structural Biology," in *Protein-Nucleic Acid Interaction*. Saeger & Heinemann, eds., Macmillan, London, 1989, Vol. 10, pp. 143-162, the contents of which are fully incorporated by reference herein. Other *tet*O sequences which may be used in the practice of the invention may be obtained from the references given in the following (Waters *et al.*, *Nucl. Acids Res. 11*:6089-6105 (1983); Postle *et al.*, *Nucl. Acids Res. 12*:4849-4863 (1984); Unger *et al.*, *Gene 31*:103-108 (1984); Unger *et al.*, *Nucl. Acids Res. 12*:7693-

7703 (1984); Tovar *et al.*, *Mol. Gen. Genet. 215*:76-80 (1988); for comparison and overview see Hillen and Wissmann in *Protein-Nucleic Acid Interaction*, Topics in Molecular and Structural Biology, Saenger and Heinemann (eds.), Macmillan, London, Vol. 10, pp. 143-162 (1989)), the disclosures of which are fully incorporated by reference herein in their entirety. One, two, three, four, five, six, seven, eight, nine or ten or more copies of the *tet* operator sequence may be employed, with a greater number of such sequences allowing an enhanced range of regulation. Multiple copies of the *tet* operator sequence provides a synergistic effect on the ability to control expression of the heterologous protein.

The polynucleotide sequence specifying the cytomegalovirus promoter may be obtained according to Boshart *et al.*, *Cell 41*:521-530 (1985), the contents of which are fully incorporated by reference herein. Preferably, positions +75 to -53 or +75 to -31 of the promoter-enhancer may be employed. The promoter may be followed by a polylinker and then by the gene coding for the tetracycline transactivator fusion protein.

The invention also relates to an autoregulatory tetracycline-regulated system for inducing gene expression in eucaryotes, wherein a second polynucleotide molecule is introduced into the host. The second polynucleotide molecule encodes a protein of interest, wherein said polynucleotide is operably linked to a minimal promoter operatively linked to at least one *tet* operator (*tet*O) sequence. The minimal promoter linked to at least one *tet*O sequence is obtained as described above with regard to the first polynucleotide molecule. The difference between the first and the second polynucleotide molecules is that the promoter may be followed by a polylinker and then by the gene encoding the protein of interest. While the luciferase gene or other reporter genes may be used to demonstrate the operability of the regulatory system, the invention is not intended to be so limited.

The invention further relates to homologous and heterologous genes involved in developmental and differentiation processes, as well as in metabolic

pathways ensuring cellular function and communication. It relates furthermore to cellular systems utilized in the production of substances of commercial interest, including, but not limited to immunoglobulins, components of the cytoskeleton, cell adhesion proteins, receptors, cytokines peptide hormones and enzymes.

The present invention also relates to eucaryotic cells transfected with the polynucleotide molecules of the present invention. In particular, the invention relates to eucaryotic cells transfected with

(a) a first polynucleotide molecule coding for a transactivator fusion protein comprising a prokaryotic *tet* repressor and a protein capable of activating transcription in eucaryotes, wherein said first polynucleotide molecule is operably linked to a minimal promoter and at least one *tet* operator sequence; and

(b) a second polynucleotide molecule coding for a protein, wherein said second polynucleotide molecule is operably linked to a minimal promoter and at least one *tet* operator sequence.

The two polynucleotide molecules may reside on the same or separate vectors. In a preferred embodiment, the first polynucleotide is integrated into the chromosome of a eucaryotic cell or transgenic animal and the second polynucleotide is introduced as part of a vector. Integration may be achieved where there is crossover at regions of homology shared between the incoming polynucleotide molecule and the particular genome.

The expression of the heterologous protein from such transfected eucaryotic cells may be tightly regulated. Unexpectedly, it has been determined that the autoregulatory expression system of the present invention may be used to induce expression by greater than 200 to 3700 fold, compared to greater than 50 to 100 fold increase observed when the constitutive expression system is used. In addition, it has been discovered that the expression system of the present invention allows one to rapidly turn on and off the expression of the heterologous gene in a reversible way. Moreover, it has been discovered that the expression system of the invention allows one to achieve a desired level of expression according to how much tetracycline or tetracycline analogue is employed. Thus,

the autoregulatory expression system of the present invention is a great advance in the art.

The invention also relates to a method to decrease or to shut off (deactivate) the expression of a protein coded for by a polynucleotide, comprising cultivating the transfected eucaryotic cells of the present invention in a medium comprising tetracycline or a tetracycline analogue. It is possible to closely control the extent of expression by carefully controlling the concentration of tetracycline or tetracycline analogue in the culture media. As little as 0.0001 μg/ml of tetracycline will begin to result in a decrease of polypeptide (luciferase) expression. At about 0.1-1.0 μg/ml the expression is essentially shut off. The concentration of tetracycline or tetracycline analog which can be used to regulate the expression level may range from about 0.0001 to about 1 μg/ml.

The invention also relates to a method to turn on (activate) or to increase the expression of a protein coded for by a polynucleotide, comprising cultivating the eucaryotic cell of the invention in a medium lacking tetracycline or a tetracycline analogue.

Media which may be used in the practice of the invention include any media which are compatible with the transfected eucaryotic cells of the present invention. Such media are commercially available (Gibco/BRL).

The invention also relates to transgenic animals comprising one or two of the polynucleotide molecules of the present invention. Such transgenic animals may be obtained, for example, by injecting the polynucleotide into a fertilized egg which is allowed to develop into an adult animal. In particular, a few hundred DNA molecules are injected into the pro-nucleus of a fertilized one cell egg. The microinjected eggs are then transferred into the oviducts of pseudopregnant foster mothers and allowed to develop. It has been reported by Brinster *et al., Proc. Natl. Acad. Sci. USA 82*:4438-4442 (1985), the contents of which are fully incorporated by reference herein, that about 25% of mice which develop will inherit one or more copies of the microinjected DNA. Alternatively, the transgenic animals may be obtained by utilizing recombinant ES cells for the

generation of the transgenes, as described by Gossler *et al.*, *Proc. Natl. Acad. Sci. USA 83*:9065-9069 (1986), the contents of which are fully incorporated by reference herein. Animals transgenic for the gene encoding a *tet*R/transcriptional activator domain fusion protein under the transcriptional control of at least one Tet-op sequences described above and/or the gene under control of this regulatory protein can be generated e.g. by the coinjection of the two polynucleotide molecules. Alternatively, independent animal lines transgenic for only one of the polynucleotides described can be generated in a first step:

(i) Animals transgenic only for the gene encoding the desired heterologous protein to be controlled by the transactivator can be screened for the desired nonactivated expression level. This includes indicator animals transgenic for a reporter gene (e.g. *cat*, *luc*, *lacZ*) under transcriptional control of the *tet*R/transcriptional activator domain fusion protein dependent minimal promoter, which are easy to screen for integration sites showing the desired, in general a low level basal expression. If advantageous, these empirically determined loci can be used subsequently for a homologous recombination approach (Mansour *et al.*, *Nature 336*:348-352 (1988)), by which the reporter gene is substituted by a respective gene of interest in the previously analyzed integration site.

(ii) Animals transgenic only for a gene encoding a *tet*R/transcriptional activator domain fusion protein can be analyzed for the desired expression pattern of the regulator protein.

Subsequently, the desired double transgenic animals are obtained by breeding the two complementary transgenic animal lines.

The Definitions

In the description that follows, a number of terms used in recombinant DNA technology are utilized extensively. In order to provide a clear and consistent understanding of the specification and claims, including the scope to be given such terms, the following definitions are provided.

Expression: Expression is the process by which a polypeptide is produced from a structural gene. The process involves transcription of the gene into mRNA and the translation of such mRNA into polypeptide(s).

Autoregulatory expression vector: It refers to the invention as described herein. A modified tTA gene called tTAk is placed under the control of Tetp (Figure 1). Tetracycline prevents tTA from binding to Tetp, preventing expression of both tTA and thus the desired protein (such as luciferase in Figure 1). This negative feedback cycle ensures that little or no tTA is produced in the presence of tetracycline, thereby reducing or eliminating possible toxic effects. When tetracycline is removed, however, this strategy predicts that tiny amounts of tTA protein (which may result from the leakiness of the minimal promoter), will bind to Tet-op and stimulate expression of the tTAk gene. A positive feedforward loop is initiated which in turn leads to higher levels of expression of tTA and thus, luciferase (Figure 1).

Optimal context for translational initiation: consists of the ATG methionine initiation codon, plus flanking nucleotides as defined by: Kozak, M., *Cell 44*:283-292 (1986). The sequence is: CC(A or G)CC**ATG**G, with the initiation codon shown in bold. This sequence provides for the most efficient initiation of translation by the translation machinery.

Promoter: A DNA sequence generally described as the region 5′ of a gene, located proximal to the start site of transcription. The transcription of an adjacent gene(s) is initiated at the promoter region. If a promoter is an inducible promoter, then the rate of transcription increases in response to an inducing agent. In contrast, the rate of transcription is not regulated by an inducing agent if the promoter is a constitutive promoter.

Inducible Minimal Promoter: It refers to the minimum number of nucleic acids from a promoter sequence, which in combination with other regulatory elements, is capable of initiating transcription. A minimal promoter, at the minimum, defines the transcription start site but by itself is not capable, if at all, of initiating transcription efficiently. The activity of such minimal

promoters depend on the binding of activators such as a tetracycline-controlled transactivator to operably link binding sites.

V(D)J Recombination: So called for the variable (V), diversity (D), and joining (J) gene segments used in recombination, it is a process by which the developing lymphocytes begin to generate their enormous range of binding specificities from a limited amount of genetic information. It is known to assemble seven different loci in developing lymphocytes: μ, κ, and λ in B cells, and α, β, γ, and δ in T cells (for reviews see Blackwell and Alt (1988), *Immunoglobulin genes*, In *Molecular Immunology*, Hames and Glover, eds. (Washington, D.C.: IRL Press), pp. 1-60; Davis and Bjorkman, *Nature 334*:395-402 (1988); Raulet, D.H., *Annu. Rev. Immunol.* 7:175-207 (1989)).

RAG-1 and RAG-2: RAG-1 (recombination activating gene-1) and RAG-2 are genes co-expressed in maturing lymphocytes. Expression of both genes is absolutely required for V(D)J recombination and lymphocyte development. When transfected together into fibroblasts, RAG-1 and RAG-2 induce V(D)J recombination activity. The RAG-1 and RAG-2 genes lie adjacent to each other in the vertebrate genome and encode unrelated proteins. Both RAG-1 and RAG-2 are conserved between species that carry out V(D)J recombination, and their expression pattern correlates precisely with that of V(D)J recombinase activity.

Founder: It is the original (first generation) transgenic animal, *i.e.* an animal carrying a transgene, which has been made by manipulating the genome of a fertilized egg and implanting the egg into a pseudopregnant animal.

Operator: It is the site on DNA at which a repressor protein binds to prevent transcription from initiating at the adjacent promoter.

Operon: is a unit of bacterial gene expression and regulation, including structural genes and control elements in DNA recognized by regulator gene product(s).

Repressor: It is a protein that binds to operator on DNA or to RNA to prevent transcription or translation, respectively.

Repression: is the ability of an organism to prevent synthesis of certain enzymes when their products are present: more generally, refers to inhibition of transcription (or translation) by binding of repressor protein to specific site on DNA (or mRNA).

Open Reading Frame (ORF): contains a series of triplets coding for amino acids without any termination codons; sequence is (potentially) translatable into protein.

Heterologous Protein: is a protein that does not naturally occur in the specific host organism in which it is present.

Unique Restriction site: refers to a single occurrence of a site on the nucleic acid that is recognized by a restriction enzyme.

Tetracycline Transactivator Fusion Protein: A hybrid fusion protein, the tetracycline transactivator (tTA), combines the *tet*R DNA binding domain with the transcriptional activation domain of VP-16, such that when tTA binds to a minimal promoter containing *tet*O sequences, transcription of the target gene is activated. Tetracycline binding to tTA prevents activation presumably by causing a conformational change in the *tetR* portion of tTA which blocks binding of tTA to *tet*O (Hinrichs, W., *et al.*, *Science 264*:418-420 (1994)); gene activation is achieved by removing tetracycline (Gossen, M. & Bujard, H., *Proc. Natl. Acad. Sci. USA 89*:5547-5551 (1992)).

Domain: of a protein is a discrete continuous part of the amino acid sequence that can be equated with a particular function.

Cloning vector: A plasmid or phage DNA or other DNA sequence which is able to replicate autonomously in a host cell, and which is characterized by one or a small number of restriction endonuclease recognition sites at which such DNA sequences may be cut in a determinable fashion without loss of an essential biological function of the vector, and into which a DNA fragment may be spliced in order to bring about its replication and cloning. The cloning vector may further contain a marker suitable for use in the identification of cells transformed with the cloning vector.

Expression vector: A vector similar to a cloning vector but which is capable of enhancing the expression of a gene which has been cloned into it, after transformation into a host. The cloned gene is usually placed under the control of (i.e., operably linked to) certain control sequences such as promoter sequences. Promoter sequences may be either constitutive or inducible.

Eucaryotic Cell: According to the invention, a eucaryotic cell may be a cell of any eucaryotic organism including, but not limited to, yeast, plant cells, insect cells, e.g. Schneider and Sf9 cells; mammalian cells, e.g. lymphoid and HeLa cells (human), NIH3T3 and embryonic stem cells (murine), and RK13 (rabbit) cells.

Recombinant Eucaryotic Host: According to the invention, a recombinant eucaryotic host may be any eucaryotic cell which contains the polynucleotide molecules of the present invention on an expression vector or cloning vector. This term is also meant to include those eucaryotic cells that have been genetically engineered to contain the desired polynucleotide molecules in the chromosome, genome or episome of that organism. Thus, the recombinant eucaryotic host cells are capable of stably or transiently expressing the proteins.

Recombinant vector: Any cloning vector or expression vector which contains the polynucleotide molecules of the invention.

Host: Any prokaryotic or eucaryotic cell that is the recipient of a replicable vector. A "host," as the term is used herein, also includes prokaryotic or eucaryotic cells that can be genetically engineered by well known techniques to contain desired gene(s) on its chromosome or genome. For examples of such hosts, *see* Sambrook *et al., Molecular Cloning: A Laboratory Manual*, Second Edition, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York (1989).

Gene: A DNA sequence that contains information needed for expressing a polypeptide or RNA molecule, including an RNA molecule which is *not* translated into polypeptide and functions as RNA, e.g., ribosomal genes.

Structural gene: A DNA sequence that is transcribed into messenger RNA (mRNA) that is then translated into a sequence of amino acids characteristic of a specific polypeptide.

Polynucleotide molecules: A polynucleotide molecule may be a polydeoxyribonucleic acid molecule (DNA) or a polyribonucleic acid molecule (RNA).

Complementary DNA (cDNA): A "complementary DNA," or "cDNA" gene includes recombinant genes synthesized by reverse transcription of mRNA and from which intervening sequences (introns) have been removed.

Fragment: A "fragment" of a polypeptide or a polynucleotide molecule is meant to refer to any polypeptide or polynucleotide subset of that molecule.

Tetracycline Analogue: A "tetracycline analogue" is any one of a number of compounds that are closely related to tetracycline and which bind to the *tet* repressor with a K_a of at least about 10^6 M^{-1}. Preferably, the tetracycline analogue binds with an affinity of about 10^9 M^{-1} or greater, e.g. $10^{11}M^{-1}$. Examples of such tetracycline analogues include, but are not limited to those disclosed by Hlavka and Boothe, "The Tetracyclines," in *Handbook of Experimental Pharmacology 78*, R.K. Blackwood *et al.* (eds.), Springer-Verlag, Berlin-New York, 1985; L.A. Mitscher, "The Chemistry of the Tetracycline Antibiotics," *Medicinal Research 9*, Dekker, New York, 1978; Noyee Development Corporation, "Tetracycline Manufacturing Processes," *Chemical Process Reviews*, Park Ridge, N.J., 2 volumes, 1969; R.C. Evans, "The Technology of the Tetracyclines," *Biochemical Reference Series 1*, Quadrangle Press, New York, 1968; and H.F. Dowling, "Tetracycline," *Antibiotics Monographs*, no. 3, Medical Encyclopedia, New York, 1955; the contents of each of which are fully incorporated by reference herein.

Comparison of the constitutive and autoregulatory inducible expression systems in cultured cells

After confirming that pcDNA-tTAk and pTet-tTAk could direct high levels of expression of luciferase activity, and that expression directed by pTet-tTAk was inducible (*see* Figure 5), the functional properties of these plasmids were compared in a more stringent assay: the ability to express high levels of the proteins encoded by the recombination activating genes RAG-1 and RAG-2 (Schatz, D. G. *et al.*, *Cell 59*:1035-1048 (1989); Oettinger, M. A. *et al.*, *Science 248*:1517-1523 (1990)). During lymphoid development, RAG-1 and RAG-2 participate in the assembly of functional immunoglobulin and T cell receptor genes from component variable (V), diversity (D) and joining (J) gene segments, a process known as V(D)J recombination. Most important for the experiments described here, RAG-1 and RAG-2 are necessary and sufficient to activate the V(D)J recombinase in non-lymphoid cells (reviewed in Schatz, D. G. *et al.*, *Annu. Rev. Immunol. 10*:359-383 (1992)), and the activity of the V(D)J recombinase can be quantitatively assayed using extrachromosomal recombination substrates (Hesse, J. E. *et al.*, *Cell 49*:775-783 (1987)).

Extensive efforts to express RAG-1 and RAG-2 in NIH3T3 fibroblast cells using a variety of promoters have revealed that it is difficult to achieve a recombination frequency (Rn) of greater than a few percent, as assayed with standard extrachromosomal recombination substrates (Sadofsky, *et al.*, *Nuc. Acids. Res. 22*:1805-1809 (1994); Sadofsky, *et al.*, *Nuc. Acids. Res. 21*:5644-5650 (1993); Cuomo and Oettinger, *Nuc. Acids. Res. 22*:1810-1814 (1994)). Only high titer RAG-retroviruses developed by others have reproducibly shown the ability to achieve an Rn as high as 10% (Silver, D. P. *et al.*, *Proc. Natl. Acad. Sci. USA 90*:6100-6104 (1993)). What is clear, however, is that Rn correlates strongly with RAG expression levels over at least three orders of magnitude (Rn from 0.01% to well above 10%; Oltz, E. M., *et al.*, *Mol. Cell. Biol. 13*(10):6223-6230 (1993)). Thus the ability to express the RAG proteins, as measured by

V(D)J recombinase activity, is an appropriate test of an inducible expression system both because of the difficulties that have been encountered in expressing the proteins and because of the sensitivity and range of the assay.

NIH3T3 fibroblast clones stably transfected with pcDNA-tTAk (17 clones) or with pTet-tTAk (10 clones) were tested for their ability to perform V(D)J recombination after transient transfection with a recombination substrate and Tetp-regulated RAG-1 and RAG-2 (Figure 2). Each clone was assayed in parallel in the presence (uninduced state) or absence (induced state) of tetracycline and the results compared to control transfections either lacking RAG-1 or RAG-2 (first sample in Figures 2A and 2B) or containing highly active, constitutive RAG expression constructs (in which RAG expression is driven by the hCMV promoter; second sample in Figures 2A and 2B). In addition, two NIH3T3 clones stably transfected with pTet-tTAk and the Tetp-regulated RAG expression vectors were assayed by transient transfection of the recombination substrate in the presence or absence of tetracycline (last two samples in Figure 2B).

The autoregulatory expression system (pTet-tTAk) represents a substantial improvement over the constitutive expression system (pcDNA-tTAk). Only 3 of 17 (18%) pcDNA-tTAk transfectants had clearly detectable levels of V(D)J recombination (Figure 2A), with the highest levels of recombination (in clone S1-17) being 3 fold that seen in the positive control with constitutively active RAG expression vectors (second sample; Figure 2A). Removal of tetracycline induced recombination in these three clones (S1-12, S1-13, S1-17) by greater than 50 to 100 fold. In contrast, 9 of 10 (90%) pTet-tTAk transfectants (Figure 2B) showed high levels of recombination (note the difference in scale between Figures 2A and 2B), with the highest levels (28% in S2-1) being nearly 50 fold higher than the positive control (Figure 2B, second sample). Inducibility in these nine clones was excellent, ranging from over 200 fold to 3700 fold. Equally high were the observed recombination frequencies achieved in clones

stably transfected with pTet-tTAk and Tetp-regulated RAG plasmids (S4-9 and S4-5; last two samples in Figure 2B).

Further characterization of the pTet-tTAk transfectant S2-6 and the pTet-tTAk + pTet-R1A/C + pTet-R2A transfectant S4-9 demonstrated that the ability to induce high levels of V(D)J recombinase activity is reproducible and that recombination decreases three fold with 0.01 μg/ml tetracycline and twenty fold with 0.1 μg/ml tetracycline. Greater than 50% cell death was observed within 10 days and a loss of detectable tTA protein was detected by 3 weeks in S2-6 cells cultured in the absence of tetracycline (Figure 6).

Figure 3A demonstrates that mRNA corresponding to the tTAk and RAG-1 and RAG-2 genes is detected in induced cell lines stably expressing tTA and stably or transiently expressing RAG-1 and/or RAG-2. Figure 3B shows that in S2-6 cells induced for 48 hours by tetracycline removal, tTA protein is easily detectable by Western blotting.

The creation of inducible transgenic mice

To assess the potential of using the autoregulatory tetracycline system in transgenic mice, the relevant portions of pTet-tTAk and pUHC13-3 were purified and co-microinjected into fertilized eggs, which were then implanted into pseudopregnant female mice. Five transgene positive founders were screened for inducibility by measurement of luciferase levels in peripheral blood mononuclear cells (PBMCs) from mice removed from tetracycline for 3 to 18 days. Three founders, #17, #19, and #20 showed high levels of luciferase activity after induction, ranging from 70-900 fold that obtained in extracts of PBMCs from transgene negative mice in the same experiments (Table 1). Founder #11 was leaky and #12 showed no inducible luciferase in PBMCs. It is presumed that variability in inducibility and leakiness of transgenes in different founders is a consequence of the site of integration and/or structure of the integrated transgenes. There was no obvious correlation between levels of luciferase

expression or leakiness in PBMCs and the copy number of the transgenes. Particularly significant was that when mouse #20 was again given water containing tetracycline for 18 days, after a previous 7 day induction in the absence of tetracycline, luciferase levels dropped essentially to background, demonstrating that transgene induction is reversible (Table 1). Germline transmission of the transgenes from founders #17 and #20, but not #19 was achieved.

Table 1 **Luciferase levels in peripheral blood mononuclear cells of transgenic and control mice**

Mouse	Transgene Copy #[a]		Days after removal of tetracycline from drinking water[b]				
	pTet-tTA	pTet-Luc	Day 0	Day 3	Day 7	Day 18	Day 7*
15	-	-	ND	50 (1)	9.4 (1)	ND	ND
21	-	-	11 (1.2)	ND	0	36 (0.5)	ND
13	-	-	ND	ND	60 (1)	69 (1)	ND
17	15	30	ND	1317 (26)	8595 (914)	ND	ND
20	80	120	ND	1137 (23)	7300 (777)	ND	88 (1.3)
11	40	20	943 (100)	ND	3983 (66)	3013 (44)	ND
19	20	40	0 (1)	ND	18250 (304)	ND	ND

[a] The approximate transgene copy number of pTet-tTA and pUHC13-3 (pTet-luc) as estimated from Southern blotting.

[b] Values represent light units (with lysis buffer background subtracted) per 10^6 cells measured in cell extracts of PBMCs from mice removed from tetracycline for the indicated number of days. Values in parentheses are the fold increase or decrease in luciferase activities relative to that in cell extracts from a transgene negative mouse in the assay performed the same day.

Day 7* denotes luciferase activity measured in cell extracts from mice in whose drinking water tetracycline was removed for 7 days and then restored for 18 days.

To analyze more carefully the inducibility of luciferase in transgenic mice, a variety of tissues and organs of second or third generation transgene positive progeny of founder #17 and #20 (backcrossed to C57Bl/6) were removed from tetracycline for 7 or 8 days and were compared to transgene identical positive progeny maintained on tetracycline. As shown in Figure 4 and Table 2, the progeny of mouse #5.1 (from Founder #17) showed luciferase activity in all organs examined. Levels of luciferase activity varied substantially between tissues, with expression consistently high in thymus and lung, and low in liver and kidney. Induction ranged from 2-fold in testes to 150-fold in thymus. Luciferase activity (10^5-$4x10^6$ rLU/mg protein) was also detected in day 17 fetal brain and liver of transgene positive mice conceived in the absence of tetracycline. Additionally, transgene positive mice conceived and maintained from gestation through 3.5 months in the absence of tetracycline continued to express optimal levels of luciferase and appeared normal. Progeny from founder #20 also showed highest levels of inducible luciferase activity in thymus and lung, although inducibility and tissue distribution of luciferase were more restricted than in the progeny of founder #17. Northern blotting demonstrated that tTA mRNA levels were clearly induced in the thymus and lung from progeny of mouse #51 after removal of tetracycline (Figure 3C). Mice removed from tetracycline for up to 6 months appear healthy, indicating that induction of the tTA protein *in vivo* is not toxic or lethal. It was also observed that mRNA hybridizes to a probe specific for the luciferase gene in thymus from induced mice.

Table 2 Average luciferase activity and fold induction in tissues of transgenic mice[a]

Tissue	Av. TG Neg.	Av. Unind.	Av. Ind.	Fold Induction
Spleen	107 (9)	684 (8)	33,180 (10)	48
Thymus	220 (9)	16,243 (8)	2,448,580 (10)	151
Lung	138 (9)	1,617 (8)	169,538 (10)	105
Liver	69 (3)	214 (6)	2,022 (8)	9
Kidney	87 (3)	361 (6)	9,440 (8)	26
Heart	0 (3)	5,971 (6)	32,540 (8)	5
Cerebrum	94 (7)	754 (6)	9,836 (8)	13
Cerebellum	91 (7)	904 (6)	67,410 (8)	75
LN	617 (2)	3,892 (4)	74,449 (5)	19
Testes	71 (2)	30,398 (2)	60,911 (3)	2

[a] The average values combine data from the experiments shown in Figure 4. The number of mice in each group is indicated in parentheses. The average fold induction for each tissue is shown. Values represent rLU/mg protein with lysis buffer background (130 rLU to 180 rLU) subtracted.

The autoregulatory system (pTet-tTAk) described here represents a substantial improvement over a constitutive expression strategy (pcDNA-tTAk) in cultured cells, in all likelihood because it prevents toxic effects of the transactivator in the uninduced state and allows for higher levels of transactivator after induction. The constitutive expression strategy is less effective in two regards: a smaller fraction of clones produce any expression at all (18% versus 90%) and induced V(D)J recombinase levels are much lower (by more than 70 fold, averaging over all clones). The kinetics of induction of gene expression with the two systems appears comparable. In preliminary experiments with the autoregulatory system, strong expression of transactivator mRNA is observed 12 hours post induction consistent with the optimal level of protein expression

observed at 24 hours with the constitutive system (Gossen, M. & Bujard, H., *Proc. Natl. Acad. Sci. USA 89*:5547-5551 (1992)). Stable transfection of pTet-tTAk should allow easy derivation of activator cell lines in which a variety of genes can be inducibly expressed by subsequent transient or stable transfection.

Previous attempts to create inducible transgenic mice using genes activated by heavy metal ions or aromatic hydrocarbons have been hampered by leakiness, relatively low levels of induction, restricted tissue specificity, and toxicity or carcinogenicity of inducing agents (Jones, S. N. *et al.*, *Nucl. Acids Res. 19*(23):6547-6551 (1991); Goodnow, C. C. *et al.*, *Nature 342*:385-391 (1989); and reviewed in Yarranton, G. T., *Curr. Opin. Biotech. 3*:506-511 (1992)). The constitutive tetracycline system has been used to create inducible transgenic mice (Furth, P. A., *et al.*, *Proc. Natl. Acad. Sci. USA 91*:9302-9306 (1994)) and avoids some of the difficulties of these earlier approaches. Assuming that equally sensitive luciferase measurement procedures were employed, the autoregulatory system provides approximately two orders of magnitude more luciferase activity in thymus (1.1×10^4 rLU/mg protein maximum with the constitutive system vs. 2.5×10^6 rLU/mg protein with the autoregulatory system) and lung (1.5×10^3 rLU/mg protein maximum with constitutive system vs. 1.7×10^5 rLU/mg protein with autoregulatory system). Additional benefits of the autoregulatory system appear to be a greater induction of luciferase activity in the thymus (150-fold vs. 67-fold), and easily detectable levels of luciferase activity in tissues which show little or no activity in the unmodified system such as lung, kidney and brain. Additionally, since activity in thymus, spleen, and lymph nodes is detected, this system might be especially suited to studies of the immune system. No gross perturbations of splenic architecture were observed in hematoxalin/eosin stained tissue sections from adult, luciferase expressing, transgenic mice maintained in the absence of tetracycline since conception. As seen with the unmodified system, leakiness varies between tissues, though it is higher in the thymus with the autoregulatory system than with the constitutive system.

By comparison to luciferase protein standards, the luciferase activity that was observed in thymus corresponds to an average of approximately 30 molecules of luciferase per cell. However, it is not known what fraction of cells express luciferase activity or how expression levels vary between expressing cells. Since induction of tTA expression in this system depends upon a low level of leakiness of the tTA transgene, it is expected that inducibility will vary with the transcriptional profiles of individual cell types and stages of differentiation. Therefore, per cell calculations of luciferase protein may under represent the actual levels induced in individual cells.

These results demonstrate that highly inducible and reversible expression from a Tetp-controlled reporter transgene can be obtained using the pTet-tTAk construct, and suggest that mice can develop normally in the presence of tetracycline and these transgenes, and that induction by removal of tetracycline does not lead to any obvious ill-effects on the mice, their ability to breed, or fetal development. Therefore, the potential toxicity of the tTA protein *in vivo* may not be a serious difficulty. Induced mice still express optimal levels of luciferase 3.5 months post tetracycline removal and remain viable at least six months in the absence of tetracycline, suggesting that transgene expression is tolerated and is not downregulated.

The pTet-tTAk system should be able to direct expression of any desired gene or genes in an inducible manner. This expression system should be widely applicable to the study of gene function in transfected cells and *in vivo*, to the creation of disease models for the testing of therapeutic agents, and to efforts to understand the development of mammalian organisms. It will be particularly useful in allowing regulated transgenic expression of genes otherwise too toxic to be tolerated by the organism during development.

Examples

The following examples are intended to further illustrate certain preferred embodiments of the invention and are not intended to be limiting in nature.

Materials and Methods

Details of Plasmid Constructions:

The plasmid pUHC 13-3 (described by Gossen & Bujard, *Proc. Natl. Acad. Sci. USA:89*:5547-5551 (1992)) is 5157 base pairs in size and has three EcoRI restriction sites (at positions 454, 667, 4036) which may be used for a diagnostic restriction digest. The plasmid consists of three main fragments: (1) pBR322-sequences including co1E1-origin of replication, β-lactamase-resistance-gene with the $P_{bla/p3}$ of Tn2661 (HincII-site and PstI-site removed); (2) the regulatory region with hCMV minimal promotor (-53 relative to start site) with heptamerized tet-operators upstream; and (3) the luciferase gene with 3'-flanking region from pSV-2-luc (de Wet, *et al.*, *Mol. Cell. Biol. 7*:725-37 (1987)). *See* Figure 12A for a map of pUHC 13-3, and Figures 12B-12F for a sequence of pUHC 13-3.

Sequences between the EcoRI and Xbal sites of pUHD15-1 (*see* Figure 11), Gossen & Bujard, *Proc. Natl. Acad. Sci. USA:89*:5547-5551 (1992), were replaced with a double-stranded oligonucleotide, whose sequence is shown in the top right of figure 7, to generate pCMV-tTAk (step 1). The inserted sequence provides the tTA gene with an optimal context for the initiation of translation, Kozac, M., *Nuc. Acids Res. 12*:857-872 (1984), inserts an amino acid into the tTA protein (alanine at position 2) and provides a unique HindIII site for subsequent cloning steps. This modified tTA gene is herein referred to as tTAk. The HindIII to BamHI fragment of pCMV-tTAk containing the tTAk gene was

then cloned into the HindIII to BamHI sites of pcDNAI-neo (Invitrogen Corporation) to generate pcDNA-tTAk (step 2 of figure 7).

The plasmid pTet-tTAk places a modified tTA gene called tTAk (abbreviation used herein for the tTA gene with a consensus kozak translation initiation site inserted therein) under control of the Tet promoter of pUHC-13-3 (Gossen & Bujard, *Proc. Natl. Acad. Sci. USA:89*:5547-5551 (1992)). The construct is therefore autoregulatory.

pTet-tTAk was constructed by first constructing a vector with the Tet promoter of pUHC-13-3 in a vector with an SV40 splice and poly-A site and then inserting tTAk between the Tet promoter and the splice/poly A. The SV40 intervening sequence is derived from the small T antigen: Mbo I (0.56mu=4100) to Mbo I (0.44mu=4710). The SV40 poly-A sequence is SV40 from BclI (0.19mu=2770) to EcoRI (0mu=1782), and contains the early polyadenylation sequence and the 3′ terminal sequence of the SV40 late region (coordinates given are for SV40). Just upstream of the splice region is 125 bp derived from the 3′ end of the bacterial CAT gene (untranslated sequences); these sequences appear to have no harmful effect, and were included only because they provide a convenient restriction site. *See* Figure 10A for a map of pTet-tTAk and Figures 10B-10G for the sequence of pTet-tTAk.

The starting plasmid for the construction of pTet-tTAk, pSplice-PA, was constructed by inserting the ScaI to EcoRI fragment of pHAV-CAT, Jones, *et al.*, *Nuc. Acids Res. 19*:6547-6551 (1991), into the XbaI site of pBKSII⁻ (Stratagene) by ligation of XbaI linkers after Klenow fill in of the EcoRI site. The pSplice-PA plasmid contains the SV40 intervening sequence derived from the small T antigen and the SV40 early polyadenylation sequence, as shown in Figure 8. The XhoI to SalI fragment of pUHC13-3, Gossen & Bujard, *Proc. Natl. Acad. Sci. USA:89*:5547-5551 (1992), which contains seven copies of the *tet* operator upstream of a minimal human cytomegalovirus (hCMV) promoter, was cloned into the unique XhoI site of pSplice-PA to yield pTet-Splice (figure 8, step 1). This *tet* operator-containing promoter is referred to herein as Tetp. pTet-Splice

contains a number of unique restriction sites for easy insertion of genes of interest between Tetp and the splice/poly A sequences. *See* Figure 9A for a map of pTet-Splice and Figures 9B-9G for the sequence of pTet-Splice. The HindIII to BamHI (blunted with Klenow) fragment of pCMV-tTAk containing the tTAk gene was then cloned into the HindIII to EcoRV sites of pTet-Splice to yield pTet-tTAk (figure 8, step 2).

The Tetp-controlled mouse RAG-1 expression construct used in these experiments was constructed by inserting the coding region of pR1A/C as a BamHI (blunted with Klenow) to XbaI fragment into the EcoRV to SpeI sites of pTet-Splice to yield pTet-R1A/C. However, the nucleic acid molecule encoding RAG-1 can be synthesized chemically using known methods in the art or can be isolated from any other source. For the complete sequence of RAG-1, see Schatz, D. G. *et al.*, *Cell 59*:1035-1048 (1989). The RAG-1 coding region of pR1A/C has been altered, as compared to full length mouse RAG-1, by deletion of amino acids 2-89 and amino acids 1009-1040, the addition of six histidines immediately following the second codon, the insertion of alanine and serine codons immediately after the histidines to introduce a site for NheI, and the addition of a consensus translation initiation context surrounding the AUG start codon. This deletion mutant of RAG-1 has significantly greater V(D)J recombinase activity than full length mouse RAG-1.

The tetracycline-controlled mouse RAG-2 expression construct used here was constructed by inserting the XhoI to XbaI fragment of pR2A-CDM8 into the SalI to SpeI sites of pTet-Splice to yield pTet-R2A. However, the nucleic acid molecule encoding RAG-2 can be synthesized chemically using known methods in the art or can be isolated from any other available source. For the complete sequence of RAG-2, see U.S.P.N. 5,159,066, issued October 27, 1992, or Oettinger, M. A. *et al.*, *Science 248*:1517-1523 (1990). The RAG-2 coding region of pR2A-CDM8 has been altered, relative to full length mouse RAG-2, by the C-terminal deletion of amino acids 492-527, the addition of a consensus translation initiation context surrounding the AUG start codon, and the insertion

of an alanine codon at position 2. This RAG-2 mutant has somewhat higher V(D)J recombinase activity than full length mouse RAG-2.

Example 1: Construction of plasmids

The EcoRI-BamHI fragment of pUHD15-1 (Gossen, M. & Bujard, H., *Proc. Natl. Acad. Sci. USA 89*:5547-5551 (1992)) containing the tTA open reading frame (ORF) was modified at its 5′ end by addition of a 30 bp oligonucleotide (SEQ ID NO:___) to provide an optimal context for translational initiation (Kozak, M., *Nucl. Acids Res. 12*:857-872 (1984)) and a unique HindIII site for subsequent cloning. The added nucleotides are those shown at the top right, Figure 7. The *number* of added nucleotides depends on whether one counts the nucleotides of the EcoRI and XbaI enzyme sites. For simplicity, the modified 5′ end of the ORF is referred to herein as a 30 bp oligo. The modified tTA gene is hereafter referred to as tTAk. The HindIII-BamHI tTAk fragment was cloned into the HindIII-BamHI sites of pcDNAI-neo (Invitrogen Corporation) to yield pcDNA-tTAk. In pcDNA-tTAk, the tTAk gene is under the transcriptional control of the enhancer and promoter sequences of the immediate early gene of human cytomegalovirus (hCMV). The plasmid pSplice-PA was constructed by inserting the SV40 small T antigen intervening sequence and the SV40 early polyadenylation sequence from pHAV-CAT (Jones, S. N. *et al.*, *Nucl. Acids Res. 19*:6547-6551 (1991)), into pBKSII$^+$ (Stratagene). The XhoI-SalI fragment of pUHC13-3 (Gossen, M. & Bujard, H., *Proc. Natl. Acad. Sci. USA 89*:5547-5551 (1992)), containing seven copies of the *tet* operator upstream of a minimal promoter (hereafter referred to as Tetp), was cloned upstream of the splice/polyA sequences of pSplice-PA to yield pTet-Splice. The tTAk gene was cloned into pTet-Splice to yield pTet-tTAk, placing the start site of transcription 143 bases upstream of the tTAk AUG (Figure 1).

The Tetp-controlled mouse RAG-1 expression construct (pTet-R1A/C) was constructed by inserting the coding region of pR1A/C into pTet-Splice. The

RAG-1 coding region of pR1A/C has been altered, as compared to full length mouse RAG-1, by small N and C terminal deletions which result in at least a two-fold increase in V(D)J recombinase activity, *i.e.* two fold increase in VDJ with R1A/C over R1A and R2A over R2. The Tetp-controlled mouse RAG-2 expression construct (pTet-R2A) was constructed by inserting the coding region of pR2A-CDM8 into pTet-Splice. This RAG-2 coding region is altered relative to full length mouse RAG-2 by a small C-terminal deletion which results in a small increase in V(D)J recombinase activity. pTet-R1 and pTet-R2 consist of the complete RAG-1 and RAG-2 ORFs, respectively, inserted into pTet-splice.

Example 2: Cell culture and derivation of transfected cell lines

Stable transfectants of pcDNA-tTAk were generated by calcium phosphate/glycerol shock transfection of 10 μg of linearized plasmid into 0.5 x 10^6 NIH 3T3 fibroblast cells as described (Schatz, D. G. *et al.*, *Cell 59*:1035-1048 (1989)) and 48 hours after transfection plating cells in 0.75 mg/ml G418 plus 0.5 μg/ml tetracycline. Single colonies picked after 12 days were expanded in 0.5 mg/ml G418, 0.5 μg/ml tetracycline.

Stable transfectants of pTet-tTAk alone or pTet-tTAk plus pTet-R1A/C plus pTet-R2A, were generated by transfecting 10 μg of each linearized plasmid with 1 μg of linearized pSV2-His, followed by selection in media containing L-histidinol but lacking histidine as described previously (Schatz, D. G. *et al.*, *Cell 59*:1035-1048 (1989)). Transfected cells were maintained in the presence of 0.5 μg/ml tetracycline, beginning at the time of transfection.

Example 3: Assay for V(D)J recombinase activity

V(D)J recombinase activity was measured using the extrachromosomal reporter plasmid pD243 (a signal joint deletion substrate) as described by others (Lewis, S. M. & Hesse, J. E., *EMBO J. 10*(12):3631-3639 (1991)). Briefly,

NIH3T3 fibroblast cell lines were transfected with 10 μg of pD243, and where indicated 6 μg of pTet-R1A/C and 4.8 μg of pTet-R2A, by the calcium phosphate/glycerol shock transfection method. Tetracycline was omitted from the culture medium after the transfection in the samples indicated "tet⁻". In other cases ("tet⁺"), cells were maintained in media containing 0.5 μg/ml tetracycline. Extrachromosomal plasmid molecules were harvested by rapid alkaline lysis of the cells 48 hours after transfection, and a small aliquot of the isolated DNA was electroporated into MC1061 bacteria. The electroporated bacteria were spread on LB agar plates containing 100 μg/ml ampicillin (A) and on plates containing 11 μg/ml chloramphenicol and 100 μg/ml ampicillin (CA). After sixteen hours of growth at 37°C the percent recombination, Rn, was calculated as the total number of CA resistant colonies divided by the total number of A resistant colonies, multiplied by 100. Greater than 99% of plasmids harvested from NIH3T3 fibroblasts 48 hours after transfection have replicated at least once (as indicated by their resistance to digestion by DpnI), demonstrating that essentially all of the harvested plasmid molecules have entered the nucleus of transfected cells and are therefore assumed to have been accessible for recombination (Lieber, M. R. *et al.*, *Genes and Devel. 1*:751-761 (1987)).

Example 4: RNA blot analysis

Electrophoresis of total cell RNA in 1-1.2% agarose/formaldehyde gels was followed by blotting to nylon membranes (Zetabind, CUNO or Genescreen Plus, NEN) and subsequent hybridization with DNA probes prepared using a random hexamer labelling kit (Boehringer Mannheim). Probes detecting RAG-1 and RAG-2 mRNA were prepared from fragments of the RAG-1 or RAG-2 coding regions (Schatz. D. G. *et al.*, *Cell 59*:1035-1048 (1989); Oettinger, M. A. *et al.*, *Science 248*:1517-1523 (1990)), respectively. The probe for actin has been described previously (Schatz, D. G. *et al.*, *Cell 59*:1035-1048 (1989)).

Example 5: Western blot analysis

Protein from 1.5 x 10^7 cells per lane was subjected to SDS-PAGE on an 8% polyacrylamide gel and electroblotted to a 0.2 micron PVDF membrane (BIO-RAD Laboratories). Membranes were blocked at room temperature (RT) overnight in a solution of 1%BSA, 0.5% gelatin, in TTBS (Tris buffered saline + 0.1% Tween-20), washed 2 X 5 minutes in TTBS and probed overnight with a monoclonal anti-*tet* R antibody (9F10)-containing hybridoma supernatant (S. Freundlieb and H. Bujard, Heidelberg, Germany), diluted 1:4 in 1%BSA in TTBS. The blots were washed 4 x 10 minutes in TTBS and tTA protein was detected by incubation for 40 minutes with goat anti-mouse antibody (1:10,000 in TTBS) (Amersham), washing in TTBS 4 x 10 minutes and TBS 2 x 10 minutes, and subsequent developing with an ECL western blotting kit (Amersham).

Example 6: Transgenic mice and assays for luciferase

Mice doubly transgenic for pTet-tTAk (XhoI to NotI fragment) and pUHC13-3 (XhoI to AseI fragment) were created by co-microinjection of gel purified DNA (in the presence of 0.5 μg/ml tetracycline) into fertilized F1 (C57BL/6 x C3H) eggs, which were then implanted into the uterus of pseudopregnant females. Pregnant females were provided with water containing 100 μg/ml tetracycline and 5% sucrose. Progeny were screened by probing Southern blots of tail DNA with tTA (761 bp XbaI-SalI) or luciferase (1365 bp HindIII-EcoRV) fragments labeled with α-^{32}P-dCTP as above. Transgene copy number was estimated by comparison of the Southern blot signal to those obtained from dilutions of plasmid DNA fragments.

Luciferase activity in tissues of transgenic mice was measured using an assay system according to the manufacturers instructions (#E1500, Promega Corporation). Peripheral blood mononuclear cells (PBMCs) (0.1-1.0 x 10^6 cells)

were lysed in 50 µl of lysis buffer for 15 minutes at room temperature, and after pelleting the insoluble material for 5 seconds at 14,000 rpm, 20 µl of the supernatant was mixed with 100 µl of luciferin reagent and the light produced in 10 seconds was measured in a luminometer (Berthold, Lumat LB9501, Germany). The number of cell equivalents of lysate in the assay was used to normalize luciferase activity between samples. Other tissues, harvested and quick frozen in liquid nitrogen, were ground to a powder with a cold mortar and pestle, placed in 100-200 µl luciferase lysis buffer, and incubated at RT for 15 minutes. Cell debris was pelleted for 10 seconds at 14,000 rpm and supernatant was stored at -70°C until analysis. 20 µl supernatant was used in luciferase assays. For normalization of luciferase activity between tissue lysates, total protein concentration in lysates was determined using a Bradford protein assay (Bio-Rad Laboratories). Samples were assayed within the linear range of the assay and only approximately 2-fold variation was observed as lysates were diluted. Firefly luciferase protein standard (Sigma) added to extracts from a variety of tissues from wild type mice showed no variation in activity.

While the foregoing invention has been described in some detail for purposes of clarity and understanding, it will be appreciated by one skilled in the art from a reading of this disclosure that various changes in form and detail can be made without departing from the true scope of the invention and appended claims. All patents and publications mentioned herein are incorporated by reference in their entirety.

What Is Claimed Is:

1. A composition of matter comprising a polynucleotide molecule encoding a tetracycline transactivator fusion protein, said protein comprising a prokaryotic *tet* repressor and a eucaryotic transcriptional activator protein, and said polynucleotide molecule being operably linked to an inducible minimal promoter, which promoter contains at least one *tet* operator sequence.

2. A composition of matter as claimed in claim 1, wherein the open reading frame of the polynucleotide molecule encoding the tetracycline transactivator fusion protein is modified at its 5′ end to provide an optimal context for translational initiation.

3. A composition of matter as claimed in claim 2, wherein the 5′ end of the open reading frame of the polynucleotide molecule encoding the tetracycline transactivator fusion protein is further modified to provide a unique restriction site.

4. A composition of matter as claimed in claim 3, wherein the unique restriction site is *Hind*III.

5. A composition of matter as claimed in claim 4, wherein the open reading frame of the polynucleotide molecule encoding the tetracycline transactivator fusion protein is modified at its 5′ end to encode an oligonucleotide identified as SEQ ID NO. -------.

6. A composition of matter as claimed in claim 1 which is DNA.

7. A composition of matter comprising a cloning vector containing the polynucleotide molecule of claim 1.

8. A composition of matter comprising a eucaryotic cell transfected with the polynucleotide molecule of claim 1.

9. A composition of matter as claimed in claim 8, wherein said eucaryotic cell is further transfected with a polynucleotide molecule encoding a heterologous protein operably linked to an inducible minimal promoter, which contains at least one *tet* operator sequence.

10. A composition of matter as claimed in claim 9, wherein at least one of the polynucleotide molecules is operably linked to a minimal promoter and seven *tet* operator sequences.

11. A method to decrease or shut off expression of a heterologous protein comprising

(a) transforming a eucaryotic cell with

(i) a first polynucleotide molecule encoding a tetracyline transactivator fusion protein, said protein comprising a prokaryotic *tet* repressor and a eucaryotic transciptional activator protein, and said polynucleotide molecule being operably linked to an incudicble minimal promoter, which promoter contains at least one *tet* operator sequence;

(ii) a second polynucleotide molecule encoding the heterologous protein. said protein being operably linked to an inducible minimal promoter, and said promoter containing at least one *tet* operator sequence; and

(b) cultivating the eucaryotic cell in a medium comprising tetracycline or a tetracycline analogue.

12. A method as claimed in claim 11, wherein the second polynucleotide molecule is operably linked to a minimal promoter and seven *tet* operator sequences.

13. A method to activate or enhance the expression of a heterologous protein comprising

(a) transforming a eucaryotic cell with

(i) a first polynucleotide molecule encoding tetracycline transactivator fusion protein, said protein comprising a prokaryotic *tet* repressor and a eucaryotic transciptional activator protein, and said polynucleotide molecule being operably linked to an inducible promoter, which promoter contains at least one *tet* operator sequence;

(ii) a second polynucleotide molecule encoding the heterologous protein, said protein being operably linked to an inducible minimal promoter, and said promoter containing at least one *tet* operator sequence; and

(b) cultivating the eucaryotic cell in a medium lacking tetracycline or a tetracycline analogue.

14. A composition of matter as claimed in claim 8 containing tetracycline in an amount sufficient to suppress binding of tetracycline transactivator fusion protein to said inducible minimal promoter.

15. A composition of matter as claimed in claim 9, wherein the polynucleotide molecule encoding a tetracycline transactivator fusion protein is expressed in an amount sufficient to drive expression of the polynucleotide molecule, encoding the heterologous protein, in the absence of tetracycline.

16. A composition of matter as claimed in claim 9 wherein tetracycline transactivator fusion protein is present in an amount sufficient to drive expression of the heterologous protein.

17. A composition of matter consisting essentially of the plasmid pTet-Splice.

18. A composition of matter consisting essentially of the plasmid pTet-tTAK.

19. A kit comprising a carrier means having in close confinement therein at least two container means, wherein a first container means contains a first polynucleotide molecule encoding a tetracycline transactivator fusion protein, said protein comprising a procaryotic *tet* repressor and a eucaryotic transcriptional activator protein, and said polynucleotide molecule being operably linked to an inducible minimal promoter, which promoter contains at least one *tet* operator sequence; and a second container means contains a second polynucleotide molecule encoding said inducible minimal promoter, which promoter contains at least one *tet* operator sequence, which *tet* operator sequence is strategically positioned for being operably linked to a heterologous polynucleotide sequence encoding a polypeptide.

20. A kit comprising a carrier means having in close confinement therein at least two container means, wherein a first container means contains a eucaryotic cell transfected with a first polynucleotide molecule encoding a tetracycline transactivator fusion protein, said protein comprising a procaryotic *tet* repressor and a eucaryotic transcriptional activator protein, and said polynucleotide molecule being operably linked to an inducible minimal promoter, which promoter contains at least one *tet* operator sequence; and a second container means contains a second polynucleotide molecule comprising an inducible minimal promoter, which promoter contains at least one *tet* operator sequence, which *tet* operator sequence is strategically positioned for being operably linked to a heterologous polynucleotide sequence encoding a heterologous polypeptide.

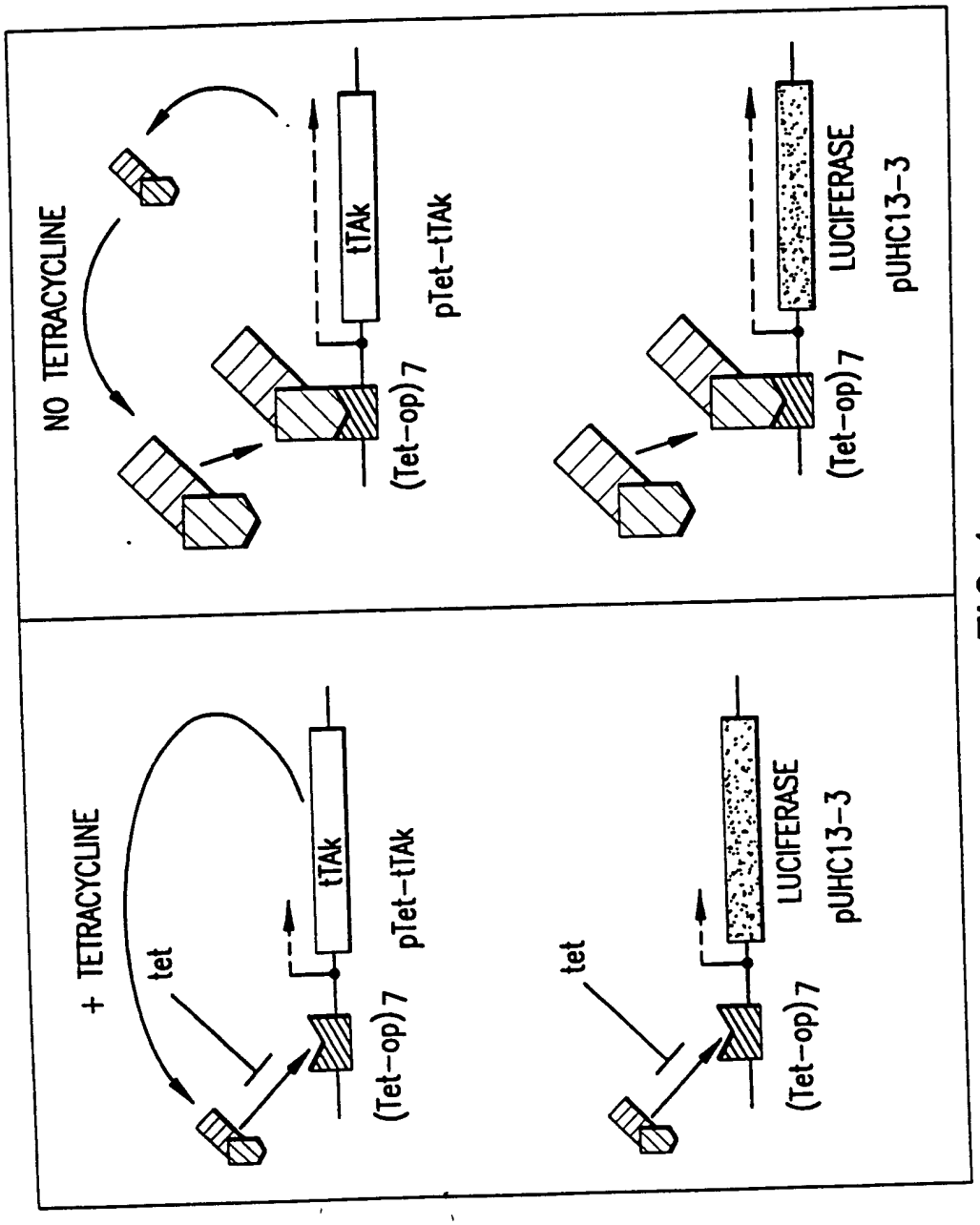

NO TETRACYCLINE
tTAk
(Tet-op) 7
pTet-tTAk
LUCIFERASE
pUHC13-3
+ TETRACYCLINE
tet

FIG. 1

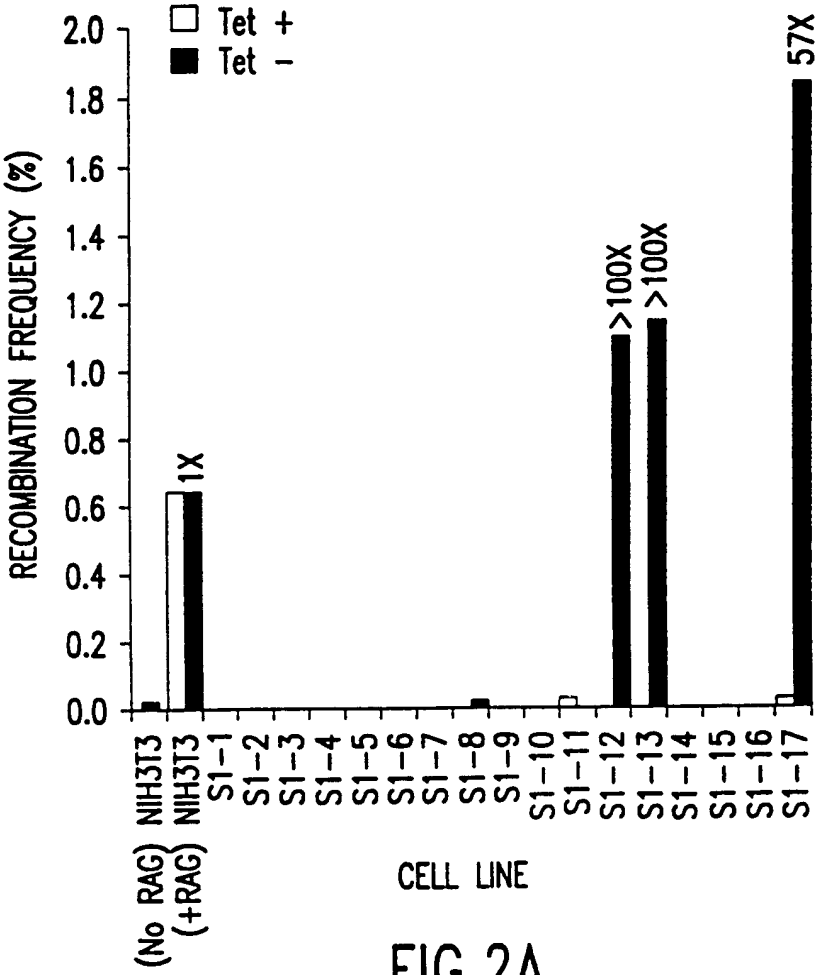

Tet +
Tet −
RECOMBINATION FREQUENCY (%)
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
1X
>100X
>100X
57X
(No RAG)
NIH3T3
(+RAG)
NIH3T3
S1-1
S1-2
S1-3
S1-4
S1-5
S1-6
S1-7
S1-8
S1-9
S1-10
S1-11
S1-12
S1-13
S1-14
S1-15
S1-16
S1-17
CELL LINE

FIG.2A

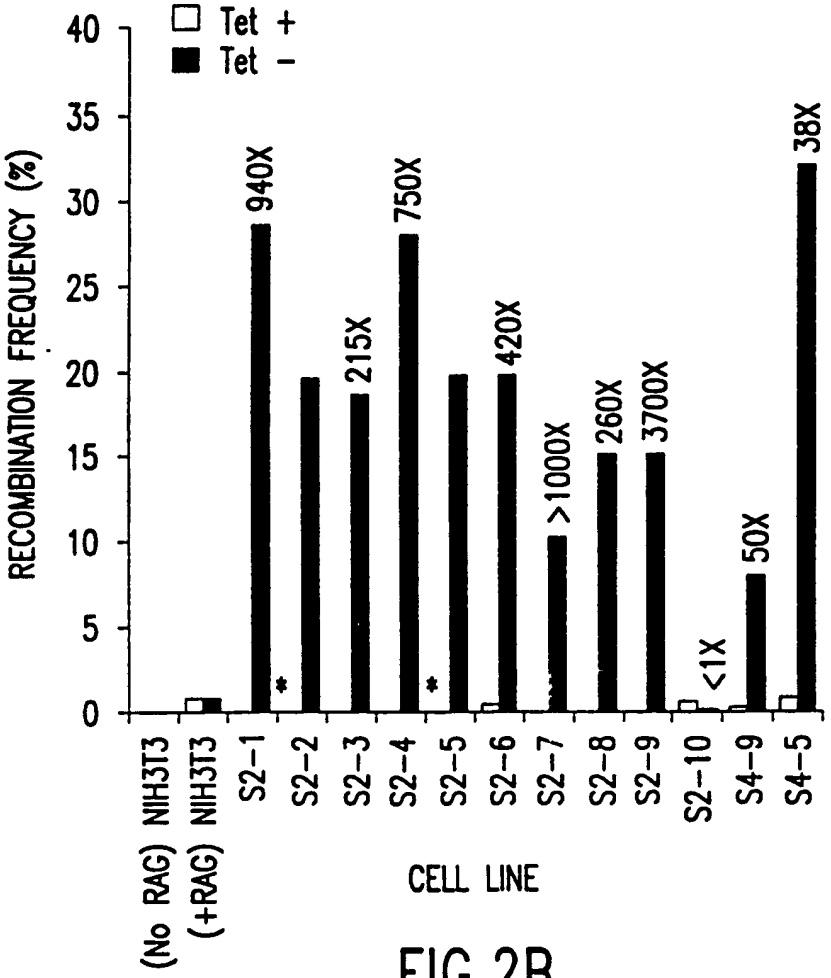

Tet +
Tet −
RECOMBINATION FREQUENCY (%)
40
35
30
25
20
15
10
5
0
940X
215X
750X
420X
>1000X
260X
3700X
<1X
50X
38X
*
*
(No RAG) NIH3T3
(+RAG) NIH3T3
S2-1
S2-2
S2-3
S2-4
S2-5
S2-6
S2-7
S2-8
S2-9
S2-10
S4-9
S4-5
CELL LINE

FIG.2B

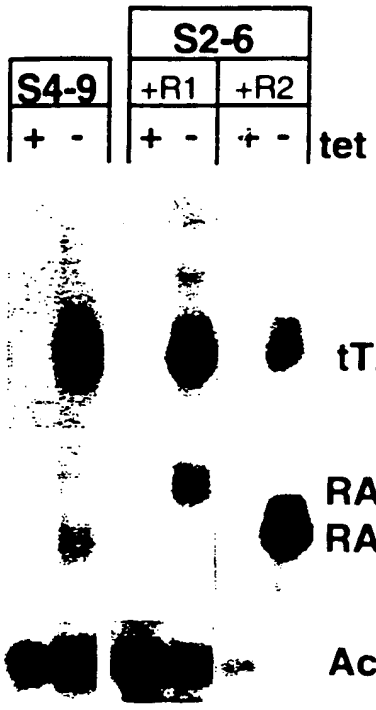


FIG. 3A

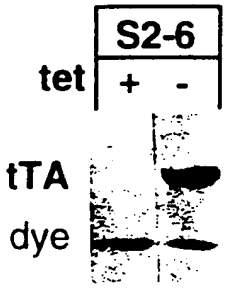


FIG. 3B

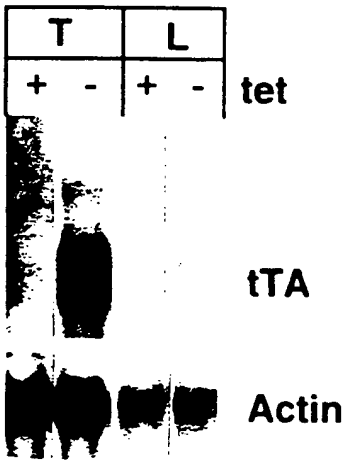


FIG. 3C

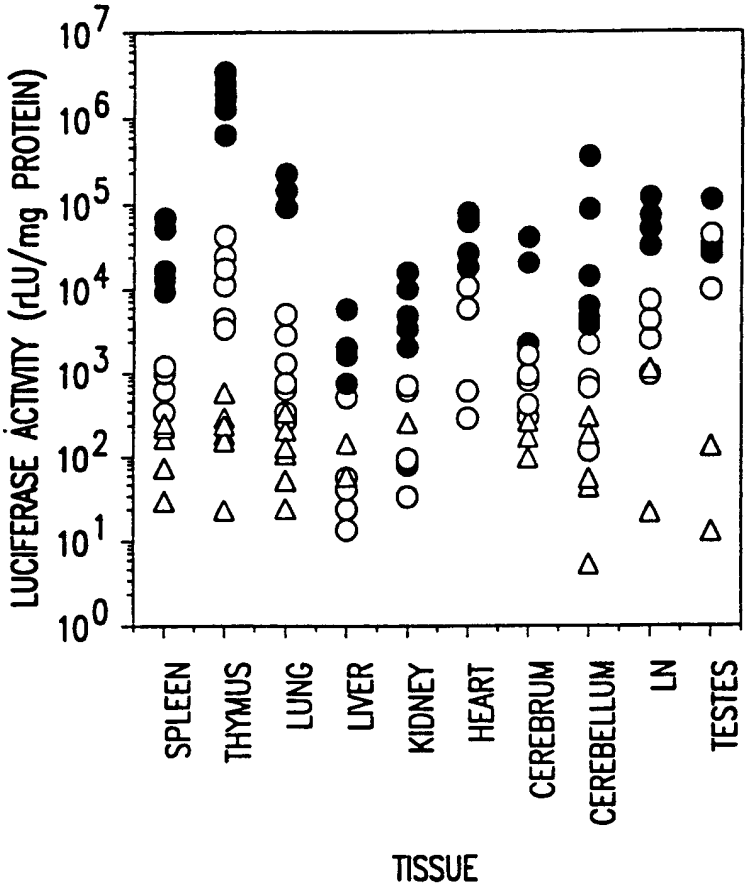

LUCIFERASE ACTIVITY (rLU/mg PROTEIN)
10^7
10^6
10^5
10^4
10^3
10^2
10^1
10^0
SPLEEN
THYMUS
LUNG
LIVER
KIDNEY
HEART
CEREBRUM
CEREBELLUM
LN
TESTES
TISSUE

FIG.4

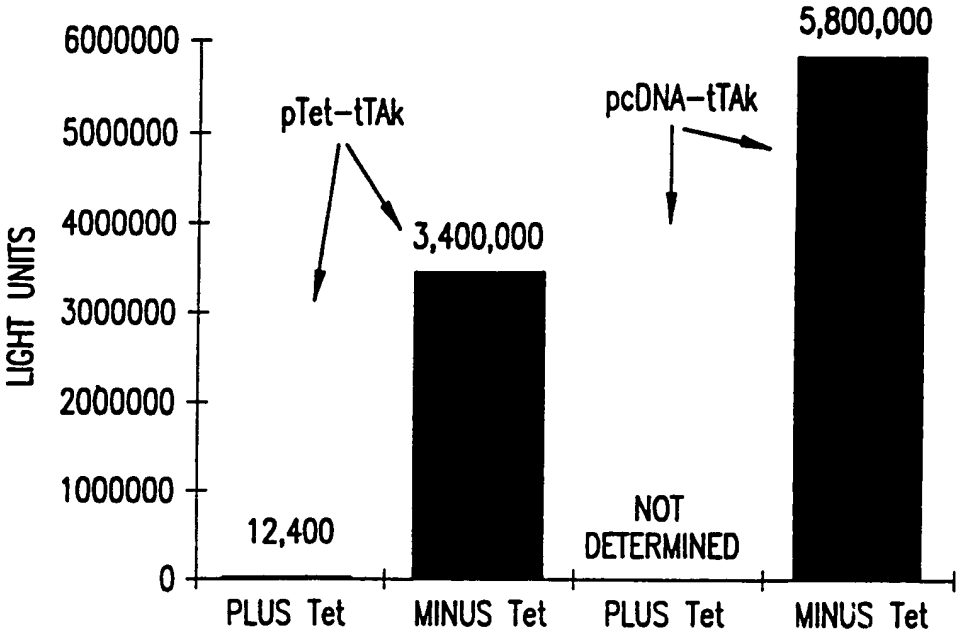
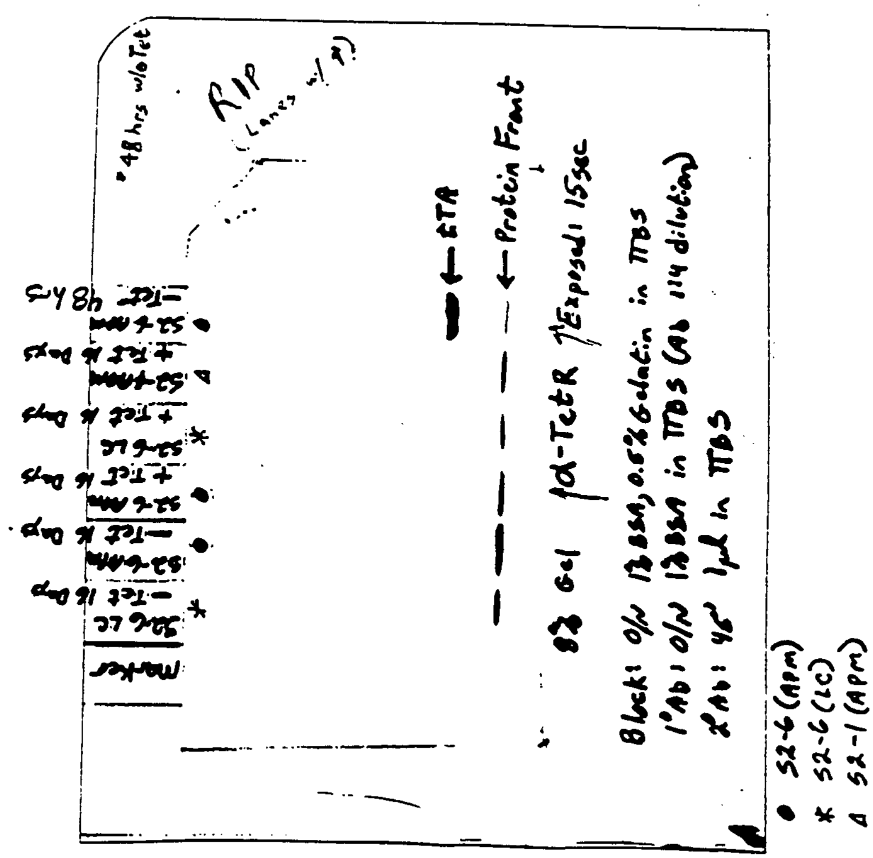

6000000
5000000
4000000
3000000
2000000
1000000
0
LIGHT UNITS
pTet-tTAk
12,400
3,400,000
pcDNA-tTAk
NOT DETERMINED
5,800,000
PLUS Tet
MINUS Tet
PLUS Tet
MINUS Tet

FIG.5



RIP
Protein Front
ETA
α-TetR
Exposed 15sec
Marker
52-6 (APM)
52-6 (LC)
52-1 (APM)

Figure 6

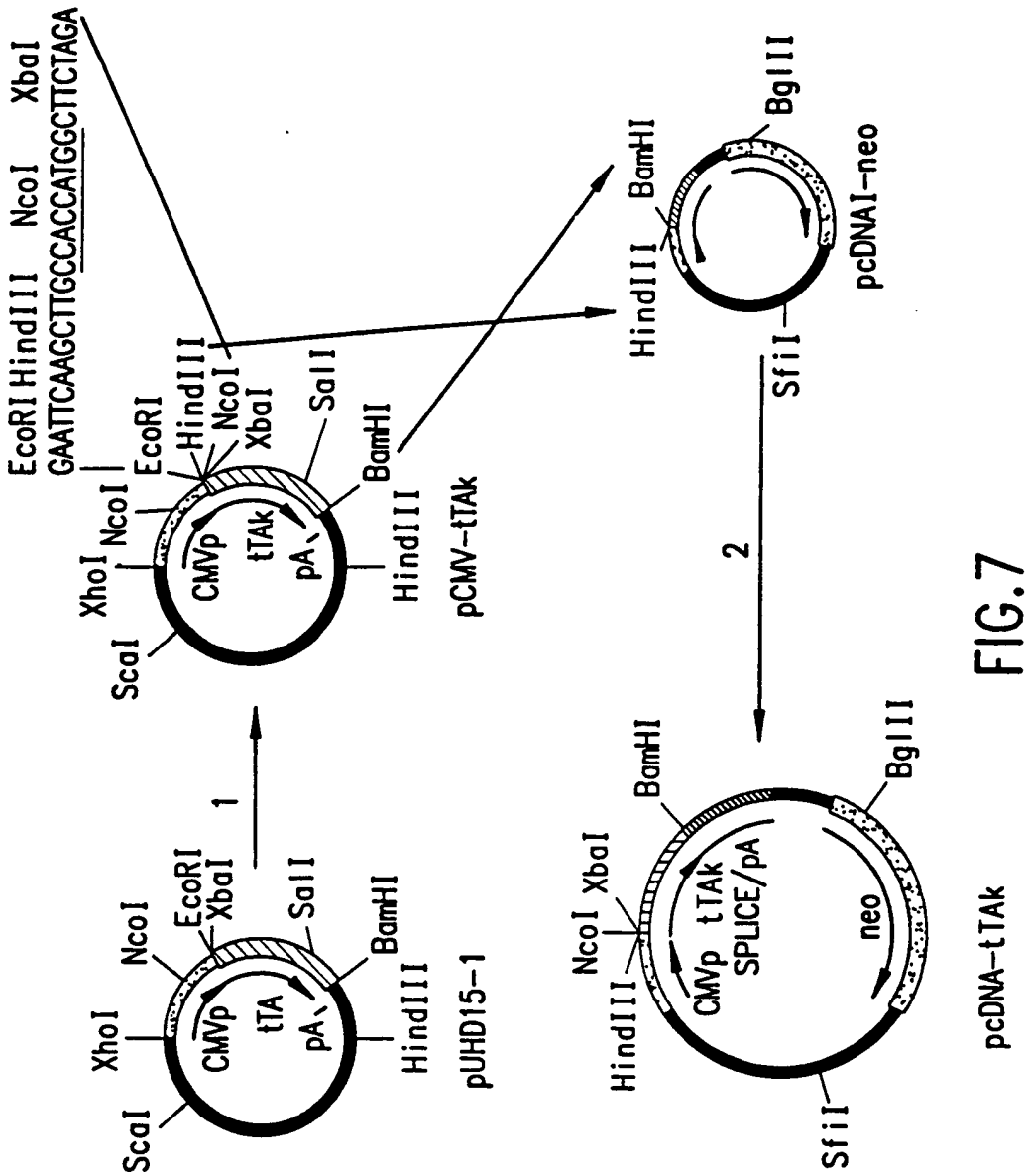

EcoRI HindIII NcoI XbaI
GAATTCAAGCTTGCCACCATGGCTTCTAGA
XhoI
NcoI
EcoRI
HindIII
NcoI
XbaI
SalI
BamHI
ScaI
CMVp
tTAk
pA
HindIII
pCMV-tTAk
1
XhoI
NcoI
EcoRI
XbaI
SalI
BamHI
ScaI
CMVp
tTA
pA
HindIII
pUHD15-1
2
HindIII
BamHI
BglII
SfiI
pcDNA1-neo
NcoI XbaI
BamHI
BglII
HindIII
CMVp tTAk
SPLICE/pA
neo
SfiI
pcDNA-tTAk

FIG.7

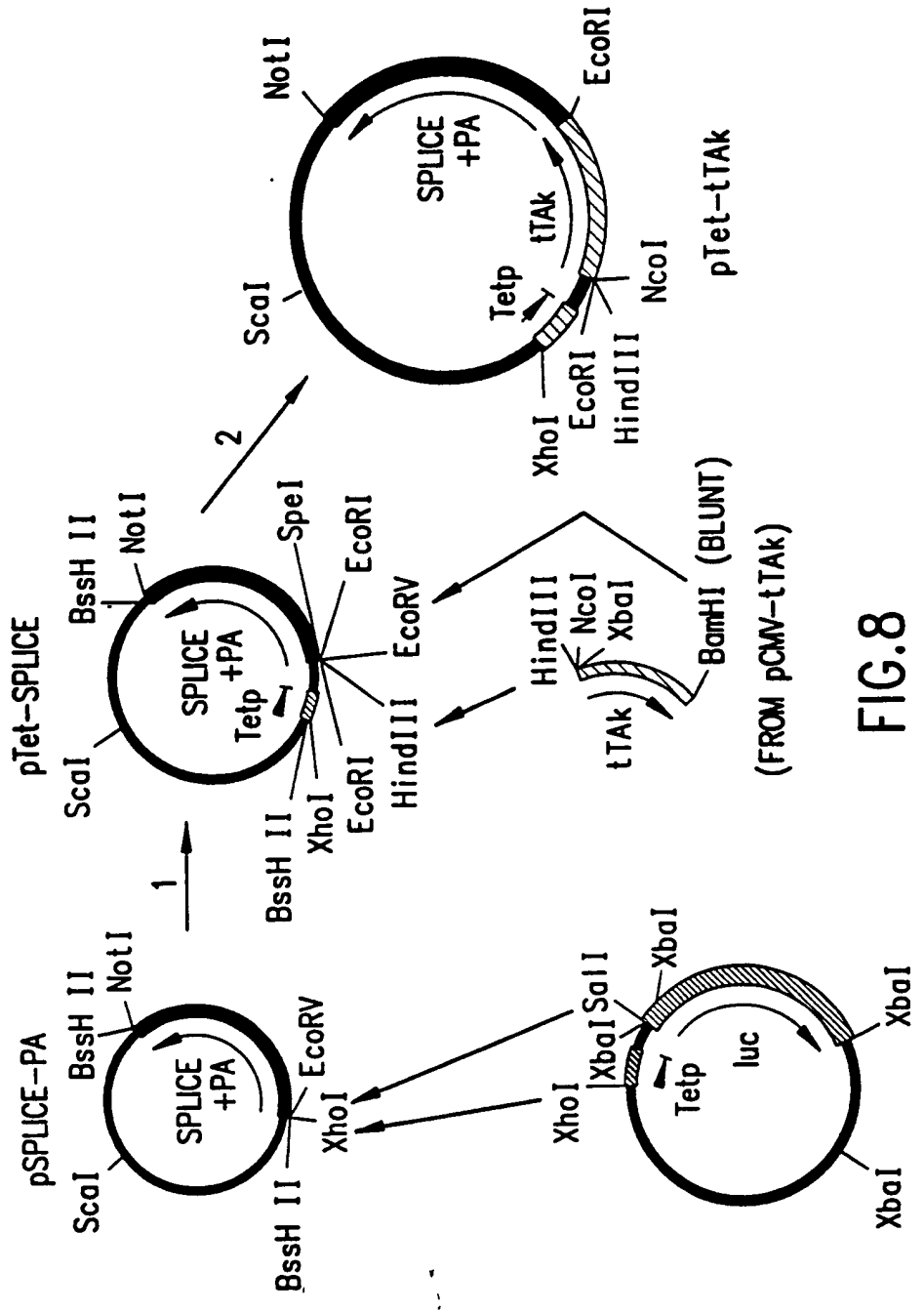

pSPLICE-PA
ScaI
BssH II
NotI
SPLICE +PA
EcoRV
XhoI
BssH II
1
pTet-SPLICE
ScaI
BssH II
NotI
SPLICE +PA
Tetp
SpeI
EcoRI
EcoRV
HindIII
XhoI
EcoRI
BssH II
2
ScaI
NotI
SPLICE +PA
EcoRI
tTAk
Tetp
NcoI
XhoI
EcoRI
HindIII
pTet-tTAk
HindIII
NcoI
XbaI
BamHI (BLUNT)
tTAk
(FROM pCMV-tTAk)
XhoI
XbaI
SalI
XbaI
Tetp
luc
XbaI
XbaI
pUHC13-3

FIG.8

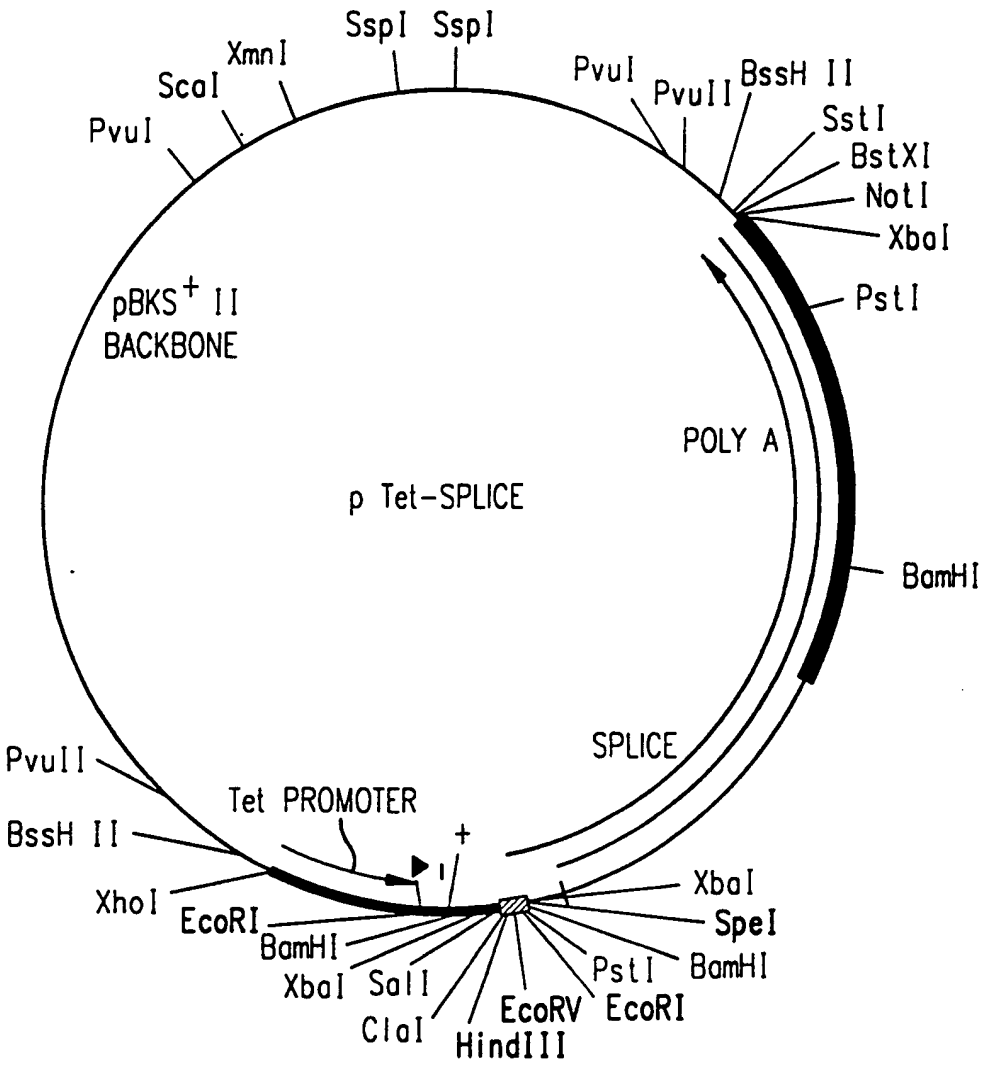

SspI SspI
XmnI
ScaI
PvuI
PvuI
PvuII
BssH II
SstI
BstXI
NotI
XbaI
PstI
pBKS+ II
BACKBONE
POLY A
p Tet-SPLICE
BamHI
SPLICE
PvuII
Tet PROMOTER
BssH II
+
XhoI
EcoRI
BamHI
XbaI
SalI
ClaI
HindIII
EcoRV
EcoRI
PstI
BamHI
XbaI
SpeI

FIG.9A

FIG. 9B

961 TGACAGTCAGCAGATGAACACTGACCACAAGGCTGTTTTGGATAAGGATAATGCTTATCCAGTGGAGTGCTGGGTTCCTG

1041 ATCCAAGTAAAAATGAAAACACTAGATATTTTGGAACCTACACAGGTGGGGAAAATGTGCCTCCTGTTTTGCACATTACT

1121 AACACAGCAACCACAGTGCTTCTTGATGAGCAGGGTGTTGGGCCCTTGTGCAAAGCTGACAGCTTGTATGTTTCTGCTGT

1201 TGACATTTGTGGGCTGTTTACCAACACTTCTGGAACACAGCAGTGGAAGGGACTTCCCAGATATTTTAAAATTACCCTTA

1281 GAAAGCGGTCTGTGAAAAACCCCTACCCAATTTCCTTTTTGTTAAGTGACCTAATTAACAGGAGGACACAGAGGGTGGAT

BamHI

1361 GGGCAGCCTATGATTGGAATGTCCTCTCAAGTAGAGGAGGTTAGGGTTTATGAGGACACAGAGGAGCTTCCTGGGGATCC

1441 AGACATGATAAGATACATTGATGAGTTTGGACAAACCACAACTAGAATGCAGTGAAAAAAATGCTTTATTTGTGAAATTT

1521 GTGATGCTATTGCTTTATTTGTAACCATTATAAGCTGCAATAAACAAGTTAACAACAACAATTGCATTCATTTTATGTTT

FIG. 9C

1601 CAGGTTCAGGGGGAGGTGTGGGAGGTTTTTTAAAGCAAGTAAAACCTCTACAAATGTGGTATGGCTGATTATGATCTTTG

1681 TGAAGGAACCTTACTTCTGTGGTGTGACATAATTGGACAAACTACCTACAGAGATTTAAAGCTCTAAGGTAAATATAAAA

1761 TTTTTAAGTGTATAATGTGTTAAACTACTGATTCTAATTGTTTGTGTATTTTAGATTCCAACCTATGGAACTGATGAATG

1841 GGAGCAGTGGTGGAATGCCTTTAATGAGGAAAACCTGTTTTGCTCAGAAGAAATGCCATCTAGTGATGATGAGGCTACTG

1921 CTGACTCTCAACATTCTACTCCTCCAAAAAAGAAGAGAAAGGTAGAAGACCCCAAGGACTTTCCTTCAGAATTGCTAAGT

2001 TTTTTGAGTCATGCTGTGTTTAGTAATAGAACTCTTGCTTGCTTTGCTATTTACACCACAAAGGAAAAAGCTGCACTGCT

2081 ATACAAGAAAATTATGGAAAAATATTCTGTAACCTTTATAAGTAGGCATAACAGTTATAATCATAACATACTGTTTTTTC

2161 TTACTCCACACAGGCATAGAGTGTCTGCTATTAATAACTATGCTCAAAAATTGTGTACCTTTAGCTTTTTAATTTGTAAA

FIG. 9D

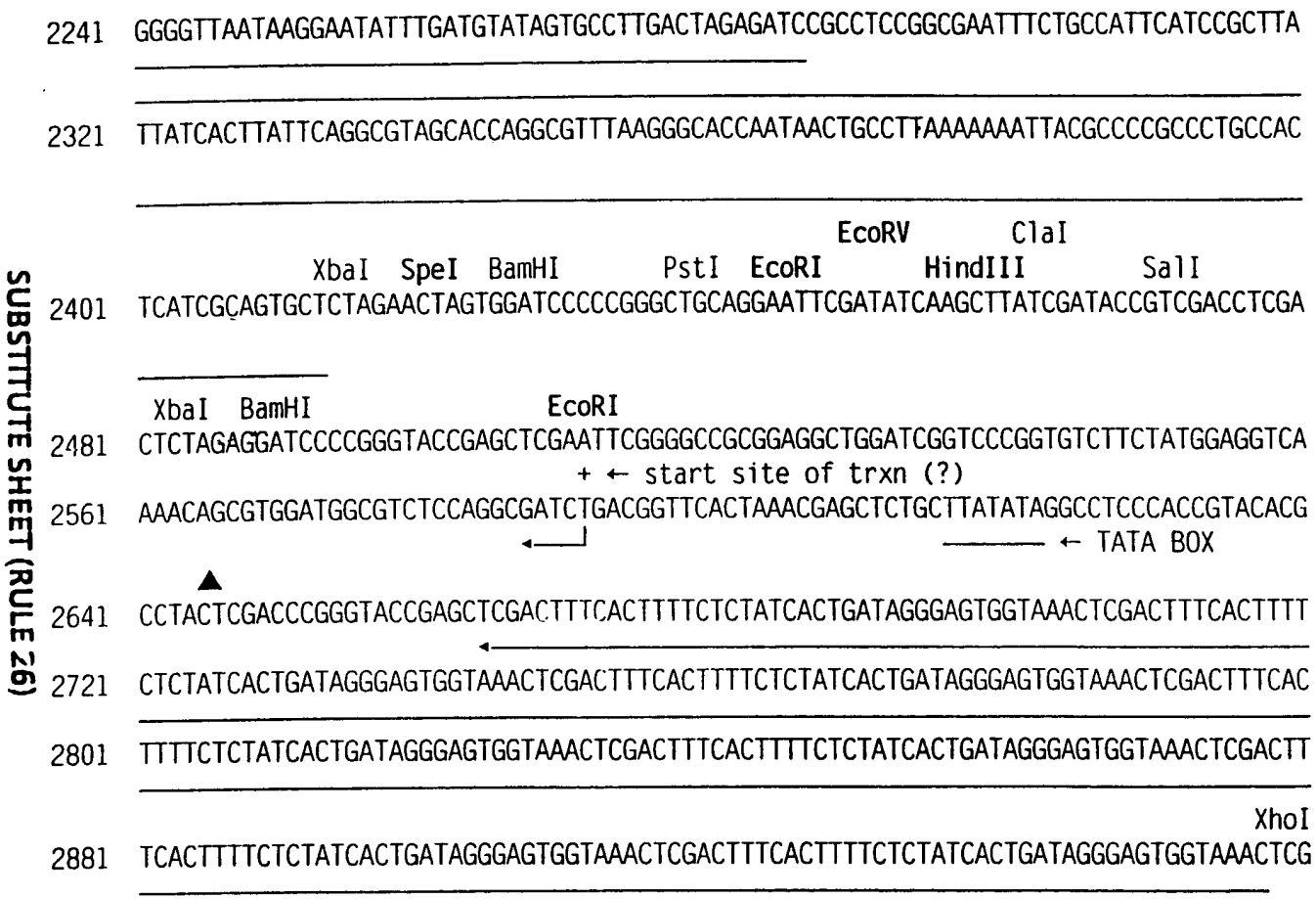


FIG. 9E

BssH II

2961 AGGGGGGGCCCGGTACCCAGCTTTTGTTCCCTTTAGTGAGGGTTAATTGCGCGCTTGGCGTAATCATGGTCATAGCTGTT
3041 TCCTGTGTGAAATTGTTATCCGCTCACAATTCCACACAACATACGAGCCGGAAGCATAAAGTGTAAAGCCTGGGGTGCCT

PvuII

3121 AATGAGTGAGCTAACTCACATTAATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTGCAT
3201 TAATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTGCGTATTGGGCGCTCTTCCGCTTCCTCGCTCACTGACTCGCTGCG
3281 CTCGGTCGTTCGGCTGCGGCGAGCGGTATCAGCTCACTCAAAGGCGGTAATACGGTTATCCACAGAATCAGGGGATAACG
3361 CAGGAAAGAACATGTGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCGCGTTGCTGGCGTTTTTCCATAGG
3441 CTCCGCCCCCCTGACGAGCATCACAAAAATCGACGCTCAAGTCAGAGGTGGCGAAACCCGACAGGACTATAAAGATACCA
3521 GGCGTTTCCCCCTGGAAGCTCCCTCGTGCGCTCTCCTGTTCCGACCCTGCCGCTTACCGGATACCTGTCCGCCTTTCTCC
3601 CTTCGGGAAGCGTGGCGCTTTCTCATAGCTCACGCTGTAGGTATCTCAGTTCGGTGTAGGTCGTTCGCTCCAAGCTGGGC
3681 TGTGTGCACGAACCCCCCGTTCAGCCCGACCGCTGCGCCTTATCCGGTAACTATCGTCTTGAGTCCAACCCGGTAAGACA
3761 CGACTTATCGCCACTGGCAGCAGCCACTGGTAACAGGATTAGCAGAGCGAGGTATGTAGGCGGTGCTACAGAGTTCTTGA
3841 AGTGGTGGCCTAACTACGGCTACACTAGAAGGACAGTATTTGGTATCTGCGCTCTGCTGAAGCCAGTTACCTTCGGAAAA
3921 AGAGTTGGTAGCTCTTGATCCGGCAAACAAACCACCGCTGGTAGCGGTGGTTTTTTTGTTTGCAAGCAGCAGATTACGCG
4001 CAGAAAAAAAGGATCTCAAGAAGATCCTTTGATCTTTTCTACGGGGTCTGACGCTCAGTGGAACGAAAACTCACGTTAAG
4081 GGATTTTGGTCATGAGATTATCAAAAAGGATCTTCACCTAGATCCTTTTAAATTAAAAATGAAGTTTTAAATCAATCTAA
4161 AGTATATATGAGTAAACTTGGTCTGACAGTTACCAATGCTTAATCAGTGAGGCACCTATCTCAGCGATCTGTCTATTTCG
4241 TTCATCCATAGTTGCCTGACTCCCCGTCGTGTAGATAACTACGATACGGGAGGGCTTACCATCTGGCCCCAGTGCTGCAA
4321 TGATACCGCGAGACCCACGCTCACCGGCTCCAGATTTATCAGCAATAAACCAGCCAGCCGGAAGGGCCGAGCGCAGAAGT
4401 GGTCCTGCAACTTTATCCGCCTCCATCCAGTCTATTAATTGTTGCCGGGAAGCTAGAGTAAGTAGTTCGCCAGTTAATAG
4481 TTTGCGCAACGTTGTTGCCATTGCTACAGGCATCGTGGTGTCACGCTCGTCGTTTGGTATGGCTTCATTCAGCTCCGGTT

PvuI

4561 CCCAACGATCAAGGCGAGTTACATGATCCCCCATGTTGTGCAAAAAAGCGGTTAGCTCCTTCGGTCCTCCGATCGTTGTC
4641 AGAAGTAAGTTGGCCGCAGTGTTATCACTCATGGTTATGGCAGCACTGCATAATTCTCTTACTGTCATGCCATCCGTAAG

FIG. 9F

```
                ScaI
4721  ATGCTTTTCTGTGACTGGTGAGTACTCAACCAAGTCATTCTGAGAATAGTGTATGCGGCGACCGAGTTGCTCTTGCCCGG
                                                    XmnI
4801  CGTCAATACGGGATAATACCGCGCCACATAGCAGAACTTTAAAAGTGCTCATCATTGGAAAACGTTCTTCGGGGCGAAAA
4881  CTCTCAAGGATCTTACCGCTGTTGAGATCCAGTTCGATGTAACCCACTCGTGCACCCAACTGATCTTCAGCATCTTTTAC
4961  TTTCACCAGCGTTTCTGGGTGAGCAAAAACAGGAAGGCAAAATGCCGCAAAAAAGGGAATAAGGGCGACACGGAAATGTT
                      SspI
5041  GAATACTCATACTCTTCCTTTTTCAATATTATTGAAGCATTTATCAGGGTTATTGTCTCATGAGCGGATACATATTTGAA
5121  TGTATTTAGAAAAATAAACAAATAGGGGTTCCGCGCACATTTCCCCGAAAAGTGCCAC
```

FIG. 9G

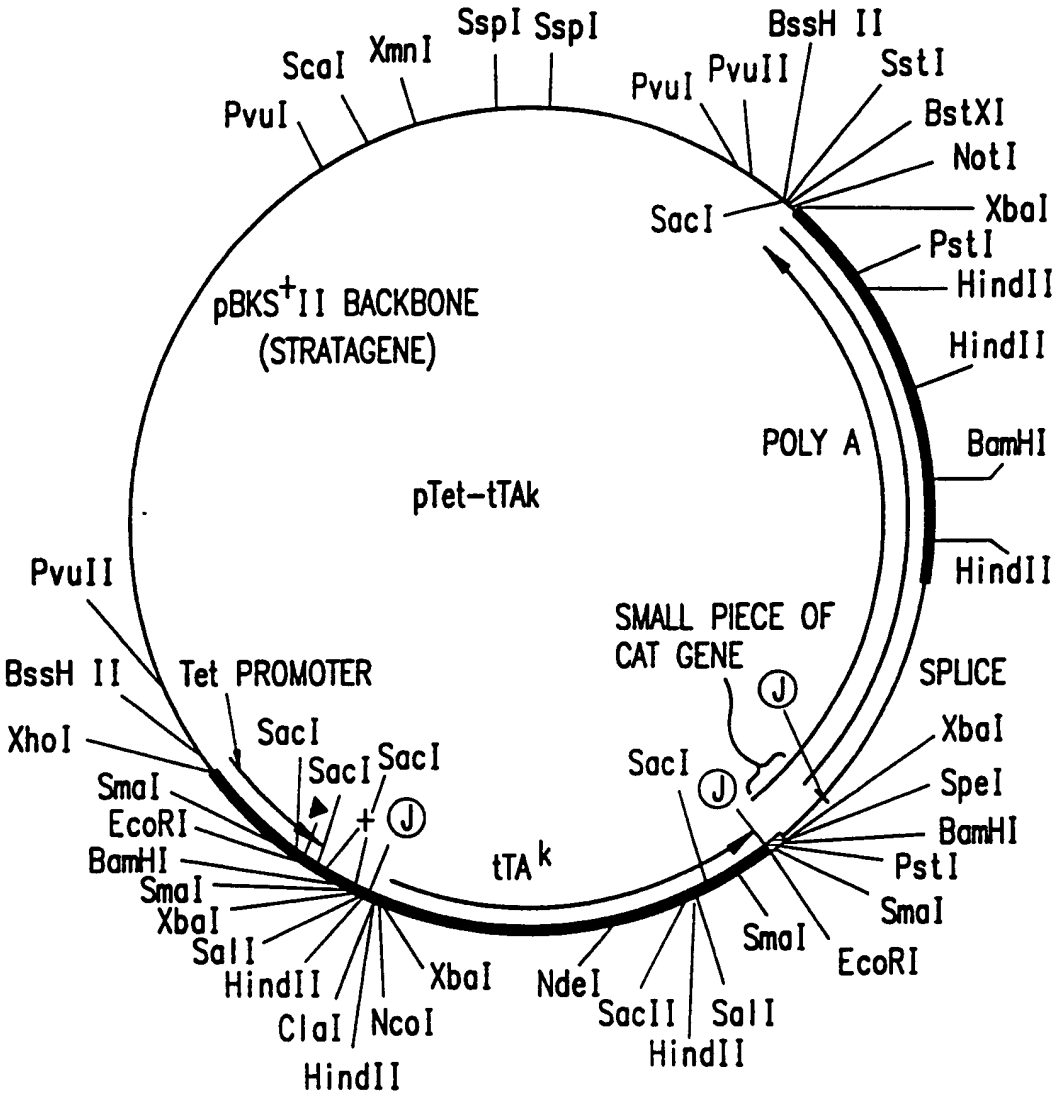

SspI SspI
XmnI
ScaI
PvuI
PvuI
PvuII
BssH II
SstI
BstXI
NotI
XbaI
SacI
PstI
HindII
HindII
BamHI
HindII
pBKS+II BACKBONE
(STRATAGENE)
POLY A
pTet-tTAk
PvuII
BssH II
Tet PROMOTER
XhoI
SacI
SacI
SacI
SmaI
EcoRI
BamHI
SmaI
XbaI
SalI
HindII
ClaI
NcoI
HindII
XbaI
tTA k
NdeI
SacII
SalI
HindII
SacI
SMALL PIECE OF
CAT GENE
SmaI
EcoRI
SPLICE
XbaI
SpeI
BamHI
PstI
SmaI

FIG.10A

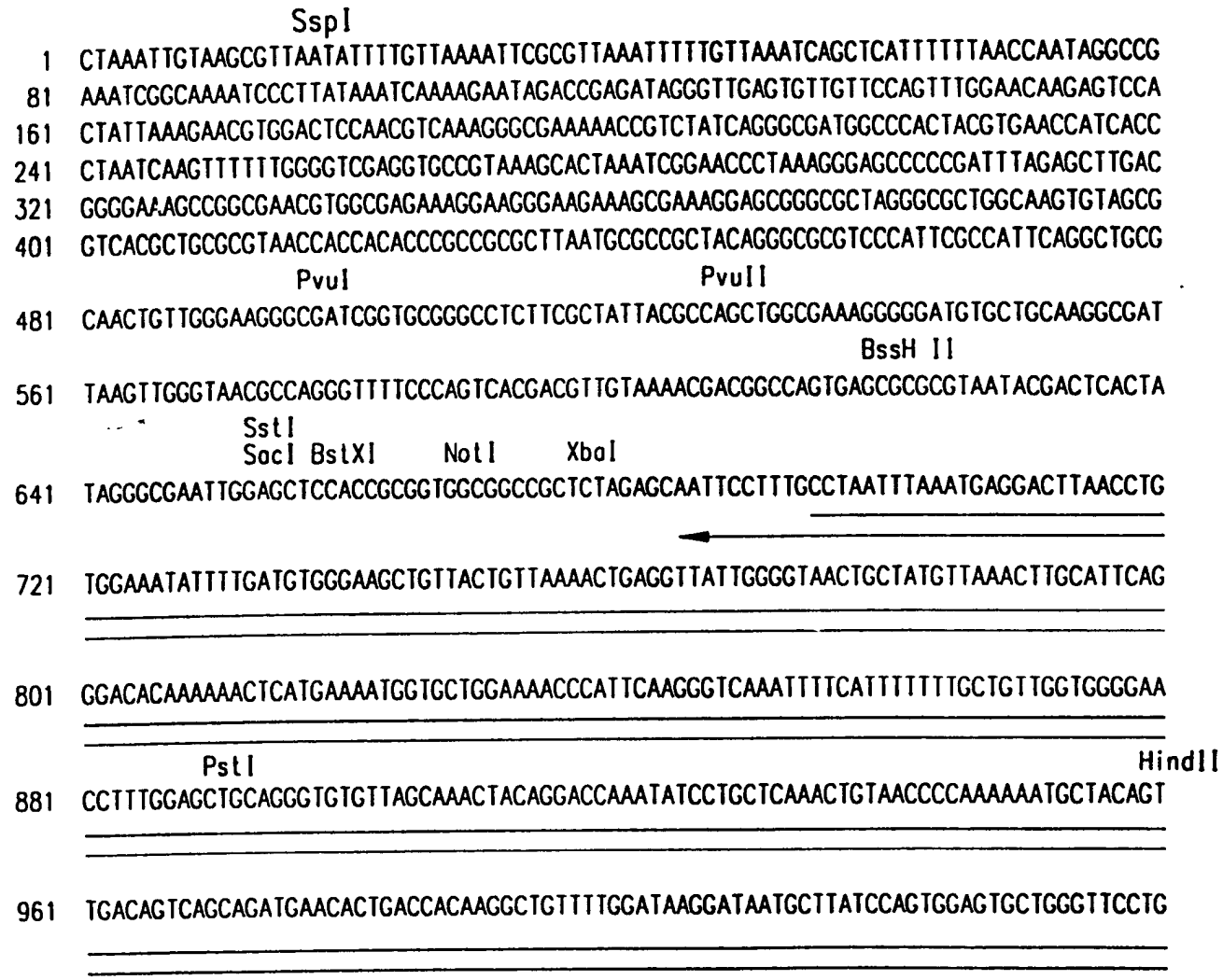

SspI
1 CTAAATTGTAAGCGTTAATATTTTGTTAAAATTCGCGTTAAATTTTTGTTAAATCAGCTCATTTTTTAACCAATAGGCCG
81 AAATCGGCAAAATCCCTTATAAATCAAAAGAATAGACCGAGATAGGGTTGAGTGTTGTTCCAGTTTGGAACAAGAGTCCA
161 CTATTAAAGAACGTGGACTCCAACGTCAAAGGGCGAAAAACCGTCTATCAGGGCGATGGCCCACTACGTGAACCATCACC
241 CTAATCAAGTTTTTTGGGGTCGAGGTGCCGTAAAGCACTAAATCGGAACCCTAAAGGGAGCCCCCGATTTAGAGCTTGAC
321 GGGGAAAGCCGGCGAACGTGGCGAGAAAGGAAGGGAAGAAAGCGAAAGGAGCGGGCGCTAGGGCGCTGGCAAGTGTAGCG
401 GTCACGCTGCGCGTAACCACCACACCCGCCGCGCTTAATGCGCCGCTACAGGGCGCGTCCCATTCGCCATTCAGGCTGCG
PvuI
PvuII
481 CAACTGTTGGGAAGGGCGATCGGTGCGGGCCTCTTCGCTATTACGCCAGCTGGCGAAAGGGGGATGTGCTGCAAGGCGAT
BssH II
561 TAAGTTGGGTAACGCCAGGGTTTTCCCAGTCACGACGTTGTAAAACGACGGCCAGTGAGCGCGCGTAATACGACTCACTA
SstI
SocI BstXI NotI XbaI
641 TAGGGCGAATTGGAGCTCCACCGCGGTGGCGGCCGCTCTAGAGCAATTCCTTTGCCTAATTTAAATGAGGACTTAACCTG
721 TGGAAATATTTTGATGTGGGAAGCTGTTACTGTTAAAACTGAGGTTATTGGGGTAACTGCTATGTTAAACTTGCATTCAG
801 GGACACAAAAAACTCATGAAAATGGTGCTGGAAAACCCATTCAAGGGTCAAATTTTCATTTTTTTGCTGTTGGTGGGGAA
PstI
HindII
881 CCTTTGGAGCTGCAGGGTGTGTTAGCAAACTACAGGACCAAATATCCTGCTCAAACTGTAACCCCAAAAAATGCTACAGT
961 TGACAGTCAGCAGATGAACACTGACCACAAGGCTGTTTTGGATAAGGATAATGCTTATCCAGTGGAGTGCTGGGTTCCTG

FIG.10B

1041 ATCCAAGTAAAAATGAAAACACTAGATATTTTGGAACCTACACAGGTGGGGAAAATGTGCCTCCTGTTTTGCACATTACT

HindII

1121 AACACAGCAACCACAGTGCTTCTTGATGAGCAGGGTGTTGGGCCCTTGTGCAAAGCTGACAGCTTGTATGTTTCTGCTGT

1201 TGACATTTGTGGGCTGTTTACCAACACTTCTGGAACACAGCAGTGGAAGGGACTTCCCAGATATTTTAAAATTACCCTTA

1281 GAAAGCGGTCTGTGAAAAACCCCTACCCAATTTCCTTTTTGTTAAGTGACCTAATTAACAGGAGGACACAGAGGGTGGAT

BamHI

1361 GGGCAGCCTATGATTGGAATGTCCTCTCAAGTAGAGGAGGTTAGGGTTTATGAGGACACAGAGGAGCTTCCTGGGGATCC

1441 AGACATGATAAGATACATTGATGAGTTTGGACAAACCACAACTAGAATGCAGTGAAAAAAATGCTTTATTTGTGAAATTT

HindII

1521 GTGATGCTATTGCTTTATTTGTAACCATTATAAGCTGCAATAAACAAGTTAACAACAACAATTGCATTCATTTTATGTTT

1601 CAGGTTCAGGGGGAGGTGTGGGAGGTTTTTTAAAGCAAGTAAAACCTCTACAAATGTGGTATGGCTGATTATGATCTTTG

FIG.10C

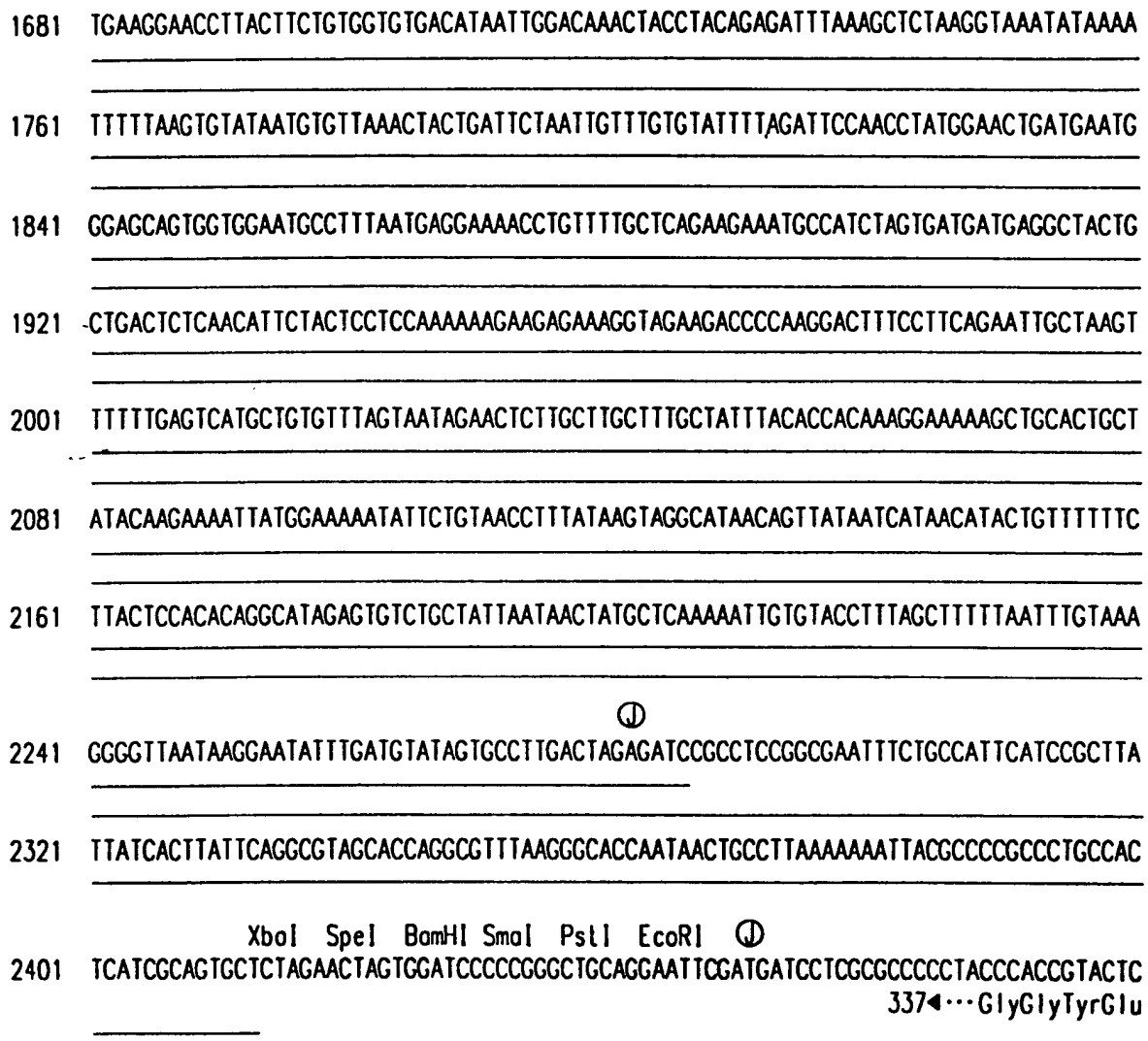

1681 TGAAGGAACCTTACTTCTGTGGTGTGACATAATTGGACAAACTACCTACAGAGATTTAAAGCTCTAAGGTAAATATAAAA
1761 TTTTTAAGTGTATAATGTGTTAAACTACTGATTCTAATTGTTTGTGTATTTTAGATTCCAACCTATGGAACTGATGAATG
1841 GGAGCAGTGGTGGAATGCCTTTAATGAGGAAAACCTGTTTTGCTCAGAAGAAATGCCATCTAGTGATGATGAGGCTACTG
1921 CTGACTCTCAACATTCTACTCCTCCAAAAAAGAAGAGAAAGGTAGAAGACCCCAAGGACTTTCCTTCAGAATTGCTAAGT
2001 TTTTTGAGTCATGCTGTGTTTAGTAATAGAACTCTTGCTTGCTTTGCTATTTACACCACAAAGGAAAAAGCTGCACTGCT
2081 ATACAAGAAAATTATGGAAAAATATTCTGTAACCTTTATAAGTAGGCATAACAGTTATAATCATAACATACTGTTTTTTC
2161 TTACTCCACACAGGCATAGAGTGTCTGCTATTAATAACTATGCTCAAAAATTGTGTACCTTTAGCTTTTTAATTTGTAAA
2241 GGGGTTAATAAGGAATATTTGATGTATAGTGCCTTGACTAGAGATCCGCCTCCGGCGAATTTCTGCCATTCATCCGCTTA
2321 TTATCACTTATTCAGGCGTAGCACCAGGCGTTTAAGGGCACCAATAACTGCCTTAAAAAAATTACGCCCCGCCCTGCCAC
2401 TCATCGCAGTGCTCTAGAACTAGTGGATCCCCCGGGCTGCAGGAATTCGATGATCCTCGCGCCCCCTACCCACCGTACTC
Xbol Spel BamHI Smal Pstl EcoRI
337◀···GlyGlyTyrGlu

FIG.10D

```
2481  GTCAATTCCAAGGGCATCGGTAAACATCTGCTCAAACTCGAAGTCGGCCATATCCAGAGCGCCGTAGGGGCGGAGTCGT
 332◄ AspIleGlyLeuAlaAspThrPheMetGlnGluPheGluPheAspAlaMetAspLeuAlaGlyTyrProAlaSerAspHi

                    SmaI
2561  GGGGGGTAAATCCCGGACCCGGGAATCCCCGTCCCCCAACATGTCCAGATCGAAATCGTCTAGCGCGTCGGCATGCGCC
 305◄ sProThrPheGlyProGlyProSerAspGlyAspGlyLeuMetAspLeuAspPheAspAspLeuAlaAspAlaHisAlaM
                                                  HindII
                                  SacI                SalI
2641  ATCGCCACGTCCTCGCCCGTCTAAGTGGAGCTCGTCCCCCAGGCTGACATCGGTCGGGGGGGCCCGTCGACAGTCTGCGCGT
 278◄ etAlaValAspGluGlyAspLeuHisLeuGluAspGlyLeuSerValAspThrProProAlaAlaThrSerLeuArgArgThr
                    SacII
2721  GTGTCCCGCGGCGAGAAGGACAGGCGCGGAGCCGCCAGCCCCGCCTCTTCGGGGGCGTCGTCCGGGAGATCGAGCA
 252◄ HisGlyAlaProLeuPheSerLeuArgProAlaAlaLeuGlyAlaGluGluProAlaAspAspAspProLeuAspLeuE
2801  GGCCCTCGATGGTAGACCCGTAATTGTTTTTCGTACGCGCGCGGCTGTACGCCGGACACCCACTTTCACATTTAAGTTGTTTT
 225◄ uGlyGluIleThrSerGlyTyrAsnAsnLysThrArgAlaArgSerTyrAlaSerGlySerGluCysLysLeuGlnLysG
                    NdeI
2881  TCTAATCCGCATATGATCAATTCAAGGCCGAATAAGAAGGCTGGCTCTGCACCTTGGTGATCAAATAATTCGATAGCTTG
 198◄ luLeuGlyCysIleIleLeuGluLeuGlyPheLeuPheAlaProGluAlaGlyGlnHisAspPheLeuGluIleAlaGln
2961  TCGTAATAATGGCGGCATACTATCAGTAGGTGTTTCCCTTTCTTTCTTTAGCGACTTGATGCTCTTGATCTTCCAATA
 172◄ ArgLeuLeuProProMetSerAspThrThrProThrArgGluGluLysAlaValGlnHisGluGlnAspGluLeuVa
3041  CGCAACCTAAAGTAAAATGCCCCACACGCCTGAGTGCATATAATGCATTCTCTAGTGAAAAACCTTGTTGGCATAAAAAG
 145◄ lCysGlyLeuThrPheHisGlyValAlaSerLeuAlaTyrLeuAlaAsnGluLeuSerPheGlyGlnGlnCysLeuPheA
3121  GCTAATTGATTTTCGAGAGTTTCATACTGTTTTTCTGTAGGCCGTGTACCTAAATGTACTTTTGCTCCATCGCCGATGACT
 118◄ laLeuGlnAsnGluLeuThrGluTyrGlnLysGluThrProArgThrGlyLeuHisValLysAlaGlyAspArgHisSer
3201  TAGTAAAGCACATCTAAAACTTTTACCGTTATTACGTAAAAAATCTTGCCAGCTTTCCCCTTCTAAAGGGCAAAAGTGAG
  92◄ LeuLeuAlaCysArgPheSerLysAlaAsnAsnArgLeuPheAspGlnTrpSerGluGlyGluLeuProCysPheHisTh
3281  TATGGTGCCTATCTAACATCTCAATGGCTAAGGCGTCGAGCAAAGCCCGCTTATTTTTTACATGCCCAATACAATGTAGGC
  65◄ rHisHisArgAspLeuMetGluIleAlaLeuAlaAspLeuLeuAlaArgLysAsnLysValHisTrpTyrLeuThrProG
```

FIG.10E

```
3361 TGCTCTACACCTAGCTTCTGGGCGAGTTTACGGGTTGTTAAACCTTCGATTCCGACCTCATTAAGCAGCTCTAATGCGCT
  38◄ InGluValGlyLeuLysGlnAlaLeuLysArgThrThrLeuGlyGluIleGlyValGluAsnLeuLeuGluLeuAlaSer
                                                                        SalI (J)
                                   XbaI    NcoI      HindIII ClaI      HindII       XbaI  BamHI
3441 GTTAATCACTTTACTTTTATCTAATCTAGAAGCCATGGTGGCAAGCTTATCGATACCGTCGACCTCGACTCTAGAGGATC
  12◄ AsnIleValLysSerLysAspLeuArgSerAla(Met) ← START OF tTA^k
     SmaI     SacI  EcoRI
3521 CCCGGGTACCGAGCTCGAATTCGGGGCCGCGGAGGCTGGATCGGTCCCGGTGTCTTCTATGGAGGTCAAAACAGCGTGGA
                    +             SacI                                        ▲   SmaI
3601 TGGCGTCTCCAGGCGATCTGACGGTTCACTAAACGAGCTCTGCTTATATAGGCCTCCCACCGTACACGCCTACTCGACCC

          SacI
3681 GGGTACCGAGCTCGACTTTCACTTTTCTCTATCACTGATAGGGAGTGGTAAACTCGACTTTCACTTTTCTCTATCACTGA

3761 TAGGGAGTGGTAAACTCGACTTTCACTTTTCTCTATCACTGATAGGGAGTGGTAAACTCGACTTTCACTTTTCTCTATCA

3841 CTGATAGGGAGTGGTAAACTCGACTTTCACTTTTCTCTATCACTGATAGGGAGTGGTAAACTCGACTTTCACTTTTCTCT

                                                                          XhoI
3921 ATCACTGATAGGGAGTGGTAAACTCGACTTTCACTTTTCTCTATCACTGATAGGGAGTGGTAAACTCGAGGGGGGGCCCG

                                BssH II
4001 GTACCCAGCTTTTGTTCCCTTTAGTGAGGGTTAATTGCGCGCTTGGCGTAATCATGGTCATAGCTGTTTCCTGTGTGAAA
4081 TTGTTATCCGCTCACAATTCCACACAACATACGAGCCGGAAGCATAAAGTGTAAAGCCTGGGGTGCCTAATGAGTGAGCT
                                                                     PvuII
4161 AACTCACATTAATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTGCATTAATGAATCGGC
4241 CAACGCGCGGGGAGAGGCGGTTTGCGTATTGGGCGCTCTTCCGCTTCCTCGCTCACTGACTCGCTGCGCTCGGTCGTTCG
4321 GCTGCGGCGAGCGGTATCAGCTCACTCAAAGGCGGTAATACGGTTATCCACAGAATCAGGGGATAACGCAGGAAAGAACA
4401 TGTGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCGCGTTGCTGGCGTTTTTCCATAGGCTCCGCCCCCCT
4481 GACGAGCATCACAAAAATCGACGCTCAAGTCAGAGGTGGCGAAACCCGACAGGACTATAAAGATACCAGGCGTTTCCCCC
```

FIG.10F

```
4561  TGGAAGCTCCCTCGTGCGCTCTCCTGTTCCGACCCTGCCGCTTACCGGATACCTGTCCGCCTTTCTCCCTTCGGGAAGCG
4641  TGGCGCTTTCTCATAGCTCACGCTGTAGGTATCTCAGTTCGGTGTAGGTCGTTCGCTCCAAGCTGGGCTGTGTGCACGAA
4721  CCCCCCGTTCAGCCCGACCGCTGCGCCTTATCCGGTAACTATCGTCTTGAGTCCAACCCGGTAAGACACGACTTATCGCC
4801  ACTGGCAGCAGCCACTGGTAACAGGATTAGCAGAGCGAGGTATGTAGGCGGTGCTACAGAGTTCTTGAAGTGGTGGCCTA
4881  ACTACGGCTACACTAGAAGGACAGTATTTGGTATCTGCGCTCTGCTGAAGCCAGTTACCTTCGGAAAAAGAGTTGGTAGC
4961  TCTTGATCCGGCAAACAAACCACCGCTGGTAGCGGTGGTTTTTTTGTTTGCAAGCAGCAGATTACGCGCAGAAAAAAAGG
5041  ATCTCAAGAAGATCCTTTGATCTTTTCTACGGGGTCTGACGCTCAGTGGAACGAAAACTCACGTTAAGGGATTTTGGTCA
5121  TGAGATTATCAAAAAGGATCTTCACCTAGATCCTTTTAAATTAAAAATGAAGTTTTAAATCAATCTAAAGTATATATGAG
5201  TAAACTTGGTCTGACAGTTACCAATGCTTAATCAGTGAGGCACCTATCTCAGCGATCTGTCTATTTCGTTCATCCATAGT
5281  TGCCTGACTCCCCGTCGTGTAGATAACTACGATACGGGAGGGCTTACCATCTGGCCCCAGTGCTGCAATGATACCGCGAG
5361  ACCCACGCTCACCGGCTCCAGATTTATCAGCAATAAACCAGCCAGCCGGAAGGGCCGAGCGCAGAAGTGGTCCTGCAACT
5441  TTATCCGCCTCCATCCAGTCTATTAATTGTTGCCGGGAAGCTAGAGTAAGTAGTTCGCCAGTTAATAGTTTGCGCAACGT
5521  TGTTGCCATTGCTACAGGCATCGTGGTGTCACGCTCGTCGTTTGGTATGGCTTCATTCAGCTCCGGTTCCCAACGATCAA
                                                                    PvuI
5601  GGCGAGTTACATGATCCCCCATGTTGTGCAAAAAAGCGGTTAGCTCCTTCGGTCCTCCGATCGTTGTCAGAAGTAAGTTG
5681  GCCGCAGTGTTATCACTCATGGTTATGGCAGCACTGCATAATTCTCTTACTGTCATGCCATCCGTAAGATGCTTTTCTGT
            ScoI
5761  GACTGGTGAGTACTCAACCAAGTCATTCTGAGAATAGTGTATGCGGCGACCGAGTTGCTCTTGCCCGGCGTCAATACGGG
                                                          XmnI
5841  ATAATACCGCGCCACATAGCAGAACTTTAAAAGTGCTCATCATTGGAAAACGTTCTTCGGGGCGAAAACTCTCAAGGATC
5921  TTACCGCTGTTGAGATCCAGTTCGATGTAACCCACTCGTGCACCCAACTGATCTTCAGCATCTTTTACTTTCACCAGCGT
6001  TTCTGGGTGAGCAAAAACAGGAAGGCAAAATGCCGCAAAAAAGGGAATAAGGGCGACACGGAAATGTTGAATACTCATAC
                  SspI
6081  TCTTCCTTTTTCAATATTATTGAAGCATTTATCAGGGTTATTGTCTCATGAGCGGATACATATTTGAATGTATTTAGAAA
6161  AATAAACAAATAGGGGTTCCGCGCACATTTCCCCGAAAAGTGCCAC
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FIG.10G

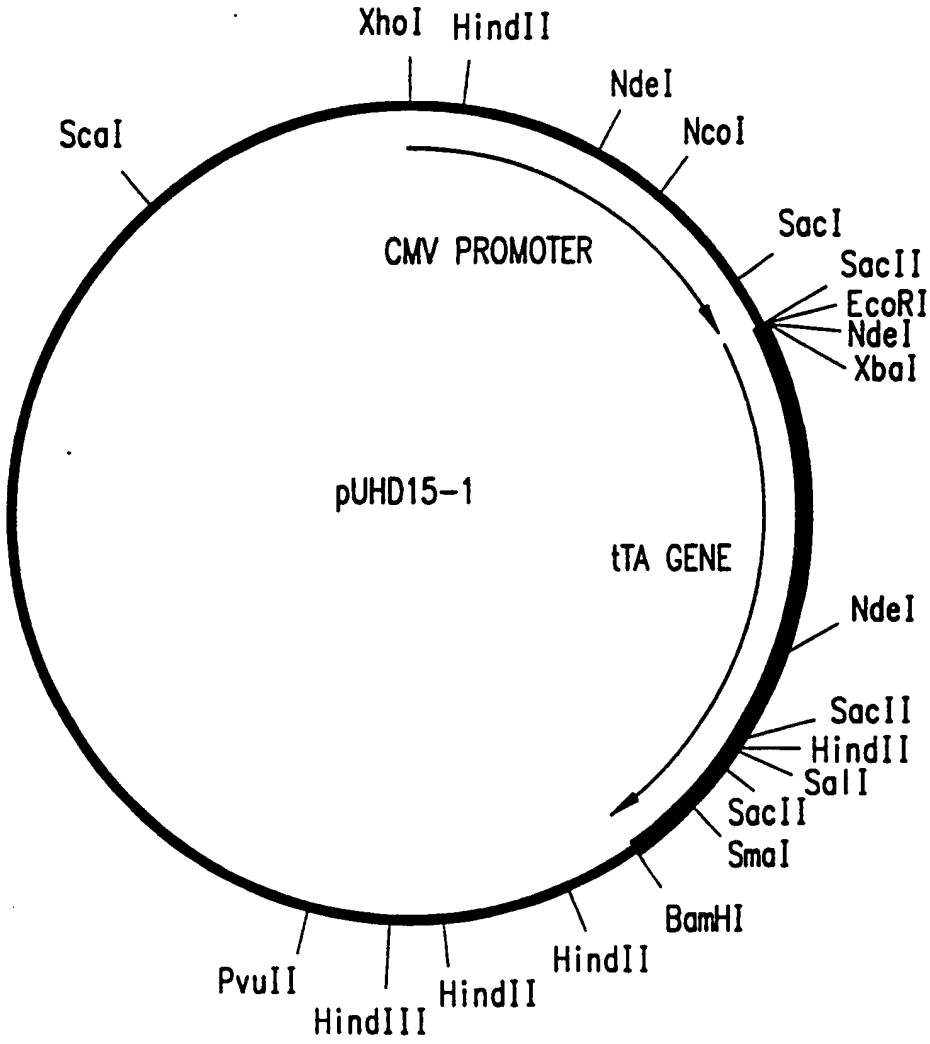
XhoI
HindII
NdeI
NcoI
ScaI
SacI
CMV PROMOTER
SacII
EcoRI
NdeI
XbaI
pUHD15-1
tTA GENE
NdeI
SacII
HindII
SalI
SacII
SmaI
BamHI
HindII
PvuII
HindIII
HindII

FIG.11A

```
      XhoI
1     CTCGAGGAGC TTGGCCCATT GCATACGTTG TATCCATATC ATAATATGTA CATTTATATT GGCTCATGTC

                HindII
71    CAACATTACC GCCATGTTGA CATTGATTAT TGACTAGTTA TTAATAGTAA TCAATTACGG GGTCATTAGT

141   TCATAGCCCA TATATGGAGT TCCGCGTTAC ATAACTTACG GTAAATGGCC CGCCTGGCTG ACCGCCCAAC

211   GACCCCCGCC CATTGACGTC AATAATGACG TATGTTCCCA TAGTAACGCC AATAGGGACT TTCCATTGAC

                                                                  NdeI
281   GTCAATGGGT GGAGTATTTA CGCTAAACTG CCCACTTGGC AGTACATCAA GTGTATCATA TGCCAAGTAC

351   GCCCCCTATT GACGTCAATG ACGGTAAATG GCCCGCCTGG CATTATGCCC AGTACATGAC CTTATGGGAC

                                                        NcoI
421   TTTCCTACTT GGCAGTACAT CTACGTATTA GTCATCGCTA TTACCATGGT GATGCGGTTT TGGCAGTACA

491   TCAATGGGCG TGGATAGCGG TTTGACTCAC GGGGATTTCC AAGTCTCCAC CCCATTGACG TCAATGGGAG

561   TTTGTTTTGG CACCAAAATC AACGGGACTT TCCAAAATGT CGTAACAACT CCGCCCCATT GACGCAAATG

                                          SacI
631   GGCGGTAGGC GTGTACGGTG GGAGGTCTAT ATAAGCAGAG CTCGTTTAGT GAACCGTCAG ATCGCCTGGA

                                                                SacII      EcoRI
701   GACGCCATCC ACGCTGTTTT GACCTCCATA GAAGACACCG GGACCGATCC AGCCTCCGCG GCCCCGAATT
      ------------------------------------------------------------------------>

      NdeI
771   CAT
```

FIG. 11B

```
        XbaI
774 ATG TCT AGA TTA GAT AAA AGT AAA GTG ATT AAC AGC GCA TTA GAG CTG CTT AAT GAG GTC
  1▸Met Ser Arg Leu Asp Lys Ser Lys Val Ile Asn Ser Ala Leu Glu Leu Leu Asn Glu Val
834 GGA ATC GAA GGT TTA ACA ACC CGT AAA CTC GCC CAG AAG CTA GGT GTA GAG CAG CCT ACA
 21▸Gly Ile Glu Gly Leu Thr Thr Arg Lys Leu Ala Gln Lys Leu Gly Val Glu Gln Pro Thr
894 TTG TAT TGG CAT GTA AAA AAT AAG CGG GCT TTG CTC GAC GCC TTA GCC ATT GAG ATG TTA
 41▸Leu Tyr Trp His Val Lys Asn Lys Arg Ala Leu Leu Asp Ala Leu Ala Ile Glu Met Leu
954 GAT AGG CAC CAT ACT CAC TTT TGC CCT TTA GAA GGG GAA AGC TGG CAA GAT TTT TTA CGT
 61▸Asp Arg His His Thr His Phe Cys Pro Leu Glu Gly Glu Ser Trp Gln Asp Phe Leu Arg
1014 AAT AAC GCT AAA AGT TTT AGA TGT GCT TTA CTA AGT CAT CGC GAT GGA GCA AAA GTA CAT
 81▸Asn Asn Ala Lys Ser Phe Arg Cys Ala Leu Leu Ser His Arg Asp Gly Ala Lys Val His
1074 TTA GGT ACA CGG CCT ACA GAA AAA CAG TAT GAA ACT CTC GAA AAT CAA TTA GCC TTT TTA
101▸Leu Gly Thr Arg Pro Thr Glu Lys Gln Tyr Glu Thr Leu Glu Asn Gln Leu Ala Phe Leu
1134 TGC CAA CAA GGT TTT TCA CTA GAG AAT GCA TTA TAT GCA CTC AGC GCT GTG GGG CAT TTT
121▸Cys Gln Gln Gly Phe Ser Leu Glu Asn Ala Leu Tyr Ala Leu Ser Ala Val Gly His Phe
1194 ACT TTA GGT TGC GTA TTG GAA GAT CAA GAG CAT CAA GTC GCT AAA GAA GAA AGG GAA ACA
141▸Thr Leu Gly Cys Val Leu Glu Asp Gln Glu His Gln Val Ala Lys Glu Glu Arg Glu Thr
1254 CCT ACT ACT GAT AGT ATG CCG CCA TTA TTA CGA CAA GCT ATC GAA TTA TTT GAT CAC CAA
161▸Pro Thr Thr Asp Ser Met Pro Pro Leu Leu Arg Gln Ala Ile Glu Leu Phe Asp His Gln
                                                    NdeI
1314 GGT GCA GAG CCA GCC TTC TTA TTC GGC CTT GAA TTG ATC ATA TGC GGA TTA GAA AAA CAA
181▸Gly Ala Glu Pro Ala Phe Leu Phe Gly Leu Glu Leu Ile Ile Cys Gly Leu Glu Lys Gln
```

FIG. 11C

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1374 CTT AAA TGT GAA AGT GGG TCC GCG TAC AGC CGC GCG CGT ACG AAA AAC AAT TAC GGG TCT
 201▸Leu Lys Cys Glu Ser Gly Ser Ala Tyr Ser Arg Ala Arg Thr Lys Asn Asn Tyr Gly Ser
1434 ACC ATC GAG GGC CTG CTC GAT CTC CCG GAC GAC GAC GCC CCC GAA GAG GCG GGG CTG GCG
 221▸Thr Ile Glu Gly Leu Leu Asp Leu Pro Asp Asp Asp Ala Pro Glu Glu Ala Gly Leu Ala
                                                                HindII
                                      SacII                     SalI
1494 GCT CCG CGC CTG TCC TTT CTC CCC GCG GGA CAC ACG CGC AGA CTG TCG ACG GCC CCC CCG
 241▸Ala Pro Arg Leu Ser Phe Leu Pro Ala Gly His Thr Arg Arg Leu Ser Thr Ala Pro Pro
                                      SacI
1554 ACC GAT GTC AGC CTG GGG GAC GAG CTC CAC TTA GAC GGC GAG GAC GTG GCG ATG GCG CAT
 261▸Thr Asp Val Ser Leu Gly Asp Glu Leu His Leu Asp Gly Glu Asp Val Ala Met Ala His
                                                                    SmaI
1614 GCG GAC GCG CTA GAC GAT TTC GAT CTG GAC ATG TTG GGG GAC GGG GAT TCC CCG GGT CCG
 281▸Ala Asp Ala Leu Asp Asp Phe Asp Leu Asp Met Leu Gly Asp Gly Asp Ser Pro Gly Pro
1674 GGA TTT ACC CCC CAC GAC TCC GCC CCC TAC GGC GCT CTG GAT ATG GCC GAC TTC GAG TTT
 301▸Gly Phe Thr Pro His Asp Ser Ala Pro Tyr Gly Ala Leu Asp Met Ala Asp Phe Glu Phe
1734 GAG CAG ATG TTT ACC GAT GCC CTT GGA ATT GAC GAG TAC GGT GGG TAG
 321▸Glu Gln Met Phe Thr Asp Ala Leu Gly Ile Asp Glu Tyr Gly Gly •••
          BamHI
1782 GGGGCGCGAG GATCCAGACA TGATAAGATA CATTGATGAG TTTGGACAAA CCACAACTAG AATGCAGTGA
1852 AAAAAATGCT TTATTTGTGA AATTTGTGAT GCTATTGCTT TATTTGTAAC CATTATAAGC TGCAATAAAC
                                                                      ————

      HindII
1922 AAGTTAACAA CAACAATTGC ATTCATTTTA TGTTTCAGGT TCAGGGGGAG GTGTGGGAGG TTTTTTAAAG
1992 CAAGTAAAAC CTCTACAAAT GTGGTATGGC TGATTATGAT CCTGCAAGCC TCGTCGTCTG GCCGGACCAC
2062 GCTATCTGTG CAAGGTCCCC GGACGCGCGC TCCATGAGCA GAGCGCCCGC CGCCGAGGCA AGACTCGGGC
```

FIG. 11D

```
                                HindII
2132 GGCGCCCTGC CCGTCCCACC AGGTCAACAG GCGGTAACCG GCCTCTTCAT CGGGAATGCG CGCGACCTTC
                                                              HindIII
2202 AGCATCGCCG GCATGTCCCC TGGCGGACGG GAAGTATCAG CTCGACCAAG·CTTGGCGAGA TTTTCAGGAG
2272 CTAAGGAAGC TAAAATGGAG AAAAAAATCA CTGGATATAC CACCGTTGAT ATATCCCAAT GGCATCGTAA
                                                                    PvuII
2342 AGAACATTTT GAGGCATTTC AGTCAGTTGC TCAATGTACC TATAACCAGA CCGTTCAGCT GCATTAATGA
2412 ATCGGCCAAC GCGCGGGGAG AGGCGGTTTG CGTATTGGGC GCTCTTCCGC TTCCTCGCTC ACTGACTCGC
2482 TGCGCTCGGT CGTTCGGCTG CGGCGAGCGG TATCAGCTCA CTCAAAGGCG GTAATACGGT TATCCACAGA
2552 ATCAGGGGAT AACGCAGGAA AGAACATGTG AGCAAAAGGC CAGCAAAAGG CCAGGAACCG TAAAAAGGCC
2622 GCGTTGCTGG CGTTTTTCCA TAGGCTCCGC CCCCCTGACG AGCATCACAA AAATCGACGC TCAAGTCAGA
2692 GGTGGCGAAA CCCGACAGGA CTATAAAGAT ACCAGGCGTT TCCCCCTGGA AGCTCCCTCG TGCGCTCTCC
2762 TGTTCCGACC CTGCCGCTTA CCGGATACCT GTCCGCCTTT CTCCCTTCGG GAAGCGTGGC GCTTTCTCAA
2832 TGCTCACGCT GTAGGTATCT CAGTTCGGTG TAGGTCGTTC GCTCCAAGCT GGGCTGTGTG CACGAACCCC
2902 CCGTTCAGCC CGACCGCTGC GCCTTATCCG GTAACTATCG TCTTGAGTCC AACCCGGTAA GACACGACTT
2972 ATCGCCACTG GCAGCAGCCA CTGGTAACAG GATTAGCAGA GCGAGGTATG TAGGCGGTGC TACAGAGTTC
3042 TTGAAGTGGT GGCCTAACTA CGGCTACACT AGAAGGACAG TATTTGGTAT CTGCGCTCTG CTGAAGCCAG
3112 TTACCTTCGG AAAAAGAGTT GGTAGCTCTT GATCCGGCAA ACAAACCACC GCTGGTAGCG GTGGTTTTTT
3182 TGTTTGCAAG CAGCAGATTA CGCGCAGAAA AAAAGGATCT CAAGAAGATC CTTTGATCTT TTCTACGGGG
3252 TCTGACGCTC AGTGGAACGA AAACTCACGT TAAGGGATTT TGGTCATGAG ATTATCAAAA AGGATCTTCA
3322 CCTAGATCCT TTTAAATTAA AAATGAAGTT TTAAATCAAT CTAAAGTATA TATGAGTAAA CTTGGTCTGA
3392 CAGTTACCAA TGCTTAATCA GTGAGGCACC TATCTCAGCG ATCTGTCTAT TTCGTTCATC CATAGTTGCC
3462 TGACTCCCCG TCGTGTAGAT AACTACGATA CGGGAGGGCT TACCATCTGG CCCCAGTGCT GCAATGATAC
3532 CGCGAGACCC ACGCTCACCG GCTCCAGATT TATCAGCAAT AAACCAGCCA GCCGGAAGGG CCGAGCGCAG
3602 AAGTGGTCCT GCAACTTTAT CCGCCTCCAT CCAGTCTATT AATTGTTGCC GGGAAGCTAG AGTAAGTAGT
3672 TCGCCAGTTA ATAGTTTGCG CAACGTTGTT GCCATTGCTA CAGGCATCGT GGTGTCACGC TCGTCGTTTG
```

FIG. 11E

```
3742 GTATGGCTTC ATTCAGCTCC GGTTCCCAAC GATCAAGGCG AGTTACATGA TCCCCCATGT TGTGCAAAAA
3812 AGCGGTTAGC TCCTTCGGTC CTCCGATCGT TGTCAGAAGT AAGTTGGCCG CAGTGTTATC ACTCATGGTT
                                                                      ScaI
3882 ATGGCAGCAC TGCATAATTC TCTTACTGTC ATGCCATCCG TAAGATGCTT TTCTGTGACT GGTGAGTACT
3952 CAACCAAGTC ATTCTGAGAA TAGTGTATGC GGCGACCGAG TTGCTCTTGC CCGGCGTCAA TACGGGATAA
4022 TACCGCGCCA CATAGCAGAA CTTTAAAAGT GCTCATCATT GGAAAACGTT CTTCGGGGCG AAAACTCTCA
4092 AGGATCTTAC CGCTGTTGAG ATCCAGTTCG ATGTAACCCA CTCGTGCACC CAACTGATCT TCAGCATCTT
4162 TTACTTTCAC CAGCGTTTCT GGGTGAGCAA AAACAGGAAG GCAAAATGCC GCAAAAAAGG GAATAAGGGC
4232 GACACGGAAA TGTTGAATAC TCATACTCTT CCTTTTTCAA TATTATTGAA GCATTTATCA GGGTTATTGT
4302 CTCATGAGCG GATACATATT TGAATGTATT TAGAAAAATA AACAAATAGG GGTTCCGCGC ACATTTCCCC
4372 GAAAAGTGCC ACCTGACGTC TAAGAAACCA TTATTATCAT GACATTAACC TATAAAAATA GGCGTATCAC
4442 GAGGCCCTTT CGTC
```

FIG. 11F

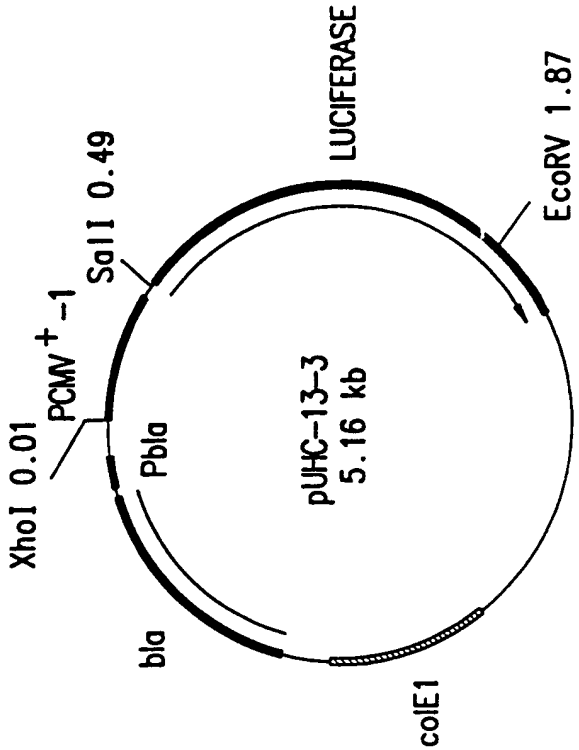
LUCIFERASE
EcoRV 1.87
SalI 0.49
PCMV+ -1
XhoI 0.01
Pbla
pUHC-13-3
5.16 kb
bla
colE1

FIG.12A

```
   EcoRI XhoI
 1 GAATTCCTCG AGTTTACCAC TCCCTATCAG TGATAGAGAA AAGTGAAAGT CGAGTTTACC ACTCCCTATC

71 AGTGATAGAG AAAAGTGAAA GTCGAGTTTA CCACTCCCTA TCAGTGATAG AGAAAAGTGA AAGTCGAGTT

141 TACCACTCCC TATCAGTGAT AGAGAAAAGT GAAAGTCGAG TTTACCACTC CCTATCAGTG ATAGAGAAAA

211 GTGAAAGTCG AGTTTACCAC TCCCTATCAG TGATAGAGAA AAGTGAAAGT CGAGTTTACC ACTCCCTATC

281 AGTGATAGAG AAAAGTGAAA GTCGAGCTCG GTACCCGGGT CGAGTAGGCG TGTACGGTGG GAGGCCTATA

351 TAAGCAGAGC TCGTTTAGTG AACCGTCAGA TCGCCTGGAG ACGCCATCCA CGCTGTTTTG ACCTCCATAG

                                        EcoRI                          XbaI  SalI
421 AAGACACCGG GACCGATCCA GCCTCCGCGG CCCCGAATTC GAGCTCGGTA CCCGGGGATC CTCTAGAGTC
491 GACCTGCAGG C
       HindIII
502 ATG CAA GCT TGG CAT TCC GGT ACT GTT GGT AAA ATG GAA GAC GCC AAA AAC ATA AAG AAA
  1▸Met Gln Ala Trp His Ser Gly Thr Val Gly Lys Met Glu Asp Ala Lys Asn Ile Lys Lys
                                 XbaI
562 GGC CCG GCG CCA TTC TAT CCT CTA GAG GAT GGA ACC GCT GGA GAG CAA CTG GAT AAG CCT
 21▸Gly Pro Ala Pro Phe Tyr Pro Leu Glu Asp Gly Thr Ala Gly Glu Gln Leu Asp Lys Pro
622 ATG AAG AGA TAC GCC CTG GTT CCT GGA ACA ATT GCT TTT ACA GAT GCA CAT ATC GAG GTG
 41▸Met Lys Arg Tyr Ala Leu Val Pro Gly Thr Ile Ala Phe Thr Asp Ala His Ile Glu Val
682 AAC ATC ACG TAC GCG GAA TAC TTC GAA ATG TCC GTT CGG TTG GCA GAA GCT GTG AAA CGA
 61▸Asn Ile Thr Tyr Ala Glu Tyr Phe Glu Met Ser Val Arg Leu Ala Glu Ala Val Lys Arg
```

FIG. 12B

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742 TAT GGG CTG AAT ACA AAT CAC AGA ATC GTC GTA TTC AGT GAA AAC TCT CTT CAA TTC TTT
 81▸Tyr Gly Leu Asn Thr Asn His Arg Ile Val Val Phe Ser Glu Asn Ser Leu Gln Phe Phe
802 ATG CCG GTG TTG GGC GCG TTA TTT ATC GGA GTT GCA GTT GCG CCC GCG AAC GAC ATT TAT
101▸Met Pro Val Leu Gly Ala Leu Phe Ile Gly Val Ala Val Ala Pro Ala Asn Asp Ile Tyr
862 AAT GAA CGT GAA TTG CTC AAC AGT ATG AAC ATT TCG CAG CCT AAC GTA GTG TTG GTT TCC
121▸Asn Glu Arg Glu Leu Leu Asn Ser Met Asn Ile Ser Gln Pro Asn Val Val Leu Val Ser
922 AAA AAG GGG TTG CAA AAA ATT TTG AAC GTG CAA AAA AAA TTA CCA ATA ATC CAG AAA ATT
141▸Lys Lys Gly Leu Gln Lys Ile Leu Asn Val Gln Lys Lys Leu Pro Ile Ile Gln Lys Ile
982 ATT ATC ATG GAT CTC AAA ACG GAT TAC CAG GGA TTT CAG TCG ATG TAC ACG TTC GTC ACA
161▸Ile Ile Met Asp Leu Lys Thr Asp Tyr Gln Gly Phe Gln Ser Met Tyr Thr Phe Val Thr
1042 TCT CAT CTA CCT CCC GGT TTT AAT GAA TAC GAT TTT GTA CCA GAG TCC TTT GAT CGT GAC
181▸Ser His Leu Pro Pro Gly Phe Asn Glu Tyr Asp Phe Val Pro Glu Ser Phe Asp Arg Asp
                          EcoRI
1102 AAA ACA ATT GCA CTG ATA ATG AAT TCC TCT GGA TCT ACT GGG TTA CCT AAG GGT GTG GCC
201▸Lys Thr Ile Ala Leu Ile Met Asn Ser Ser Gly Ser Thr Gly Leu Pro Lys Gly Val Ala
1162 CTT CCG CAT AGA ACT GCC TGC GTC AGA TTC TCG CAT GCC AGA GAT CCT ATT TTT GGC AAT
221▸Leu Pro His Arg Thr Ala Cys Val Arg Phe Ser His Ala Arg Asp Pro Ile Phe Gly Asn
1222 CAA ATC ATT CCG GAT ACT GCG ATT TTA AGT GTT GTT CCA TTC CAT CAC GGT TTT GGA ATG
241▸Gln Ile Ile Pro Asp Thr Ala Ile Leu Ser Val Val Pro Phe His His Gly Phe Gly Met
1282 TTT ACT ACA CTC GGA TAT TTG ATA TGT GGA TTT CGA GTC GTC TTA ATG TAT AGA TTT GAA
261▸Phe Thr Thr Leu Gly Tyr Leu Ile Cys Gly Phe Arg Val Val Leu Met Tyr Arg Phe Glu
1342 GAA GAG CTG TTT TTA CGA TCC CTT CAG GAT TAC AAA ATT CAA AGT GCG TTG CTA GTA CCA
281▸Glu Glu Leu Phe Leu Arg Ser Leu Gln Asp Tyr Lys Ile Gln Ser Ala Leu Leu Val Pro
1402 ACC CTA TTT TCA TTC TTC GCC AAA AGC ACT CTG ATT GAC AAA TAC GAT TTA TCT AAT TTA
301▸Thr Leu Phe Ser Phe Phe Ala Lys Ser Thr Leu Ile Asp Lys Tyr Asp Leu Ser Asn Leu
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FIG. 12C

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1462 CAC GAA ATT GCT TCT GGG GGC GCA CCT CTT TCG AAA GAA GTC GGG GAA GCG GTT GCA AAA
 321▸His Glu Ile Ala Ser Gly Gly Ala Pro Leu Ser Lys Glu Val Gly Glu Ala Val Ala Lys
1522 CGC TTC CAT CTT CCA GGG ATA CGA CAA GGA TAT GGG CTC ACT GAG ACT ACA TCA GCT ATT
 341▸Arg Phe His Leu Pro Gly Ile Arg Gln Gly Tyr Gly Leu Thr Glu Thr Thr Ser Ala Ile
1582 CTG ATT ACA CCC GAG GGG GAT GAT AAA CCG GGC GCC GTC GGT AAA GTT GTT CCA TTT TTT
 361▸Leu Ile Thr Pro Glu Gly Asp Asp Lys Pro Gly Ala Val Gly Lys Val Val Pro Phe Phe
1642 GAA GCG AAG GTT GTG GAT CTG GAT ACC GGG AAA ACG CTG GGC GTT AAT CAG AGA GGC GAA
 381▸Glu Ala Lys Val Val Asp Leu Asp Thr Gly Lys Thr Leu Gly Val Asn Gln Arg Gly Glu
1702 TTA TGT GTC AGA GGA CCT ATG ATG TCC GGT TAT GTA AAC AAT CCG CAA GCG ACC AAC
 401▸Leu Cys Val Arg Gly Pro Met Met Met Ser Gly Tyr Val Asn Asn Pro Gln Ala Thr Asn
1762 GCC TTG ATT GAC AAG GAT GGA TGG CTA CAT TCT GGA GAC ATA GCT TAC TGG GAC GAA GAC
 421▸Ala Leu Ile Asp Lys Asp Gly Trp Leu His Ser Gly Asp Ile Ala Tyr Trp Asp Glu Asp
1822 GAA CAC TTC ATA GTT GAC CGC TTG AAG TCT TTA ATT AAA TAC AAA GGA TAT CAG GTG
 441▸Glu His Phe Phe Ile Val Asp Arg Leu Lys Ser Leu Ile Lys Tyr Lys Gly Tyr Gln Val
1882 GCC CCC GCT GAA TTG GAA TCG ATA TTG TTA CAA CAC CCC AAC ATC TTC GAC GCG GGC GTG
 461▸Ala Pro Ala Glu Leu Glu Ser Ile Leu Leu Gln His Pro Asn Ile Phe Asp Ala Gly Val
1942 GCA GGT CTT CCC GAC GAT GAC GCC GGT GAA CTT CCC GCC GCC GTT GTT TTG GAG CAC
 481▸Ala Gly Leu Pro Asp Asp Asp Ala Gly Glu Leu Pro Ala Ala Val Val Leu Glu His
2002 GGA AAG ACG ATG ACG GAA AAA GAG ATC GTG GAT TAC GTC GCC AGT CAA GTA ACA ACC GCC
 501▸Gly Lys Thr Met Thr Glu Lys Glu Ile Val Asp Tyr Val Ala Ser Gln Val Thr Thr Ala
2062 AAA AAG TTG CGC GGA GGA GTT GTG TTT GTG GAC GAA GTA CCG AAA GGT CTT ACC GGA AAA
 521▸Lys Lys Leu Arg Gly Gly Val Val Phe Val Asp Glu Val Pro Lys Gly Leu Thr Gly Lys
2122 CTC GAC GCA AGA AAA ACT AGA GAG ATC CTC ATA AAG GCC AAG AAG GGC GGA AAG TCC AAA
 541▸Leu Asp Ala Arg Lys Thr Arg Glu Ile Leu Ile Lys Ala Lys Lys Gly Gly Lys Ser Lys
2182 TTG TAA
 561▸Leu •••
```

FIG. 12D

XbaI

2188 AATGTAACTG TATTCAGCGA TGACGAAATT CTTAGCTATT GTAATGACTC TAGAGGATCT TTGTGAAGGA
2258 ACCTTACTTC TGTGGTGTGA CATAATTGGA CAAACTACCT ACAGAGATTT AAAGCTCTAA GGTAAATATA
2328 AAATTTTTAA GTGTATAATG TGTTAAACTA CTGATTCTAA TTGTTTGTGT ATTTTAGATT CCAACCTATG
2398 GAACTGATGA ATGGGAGCAG TGGTGGAATG CCTTTAATGA GGAAAACCTG TTTTGCTCAG AAGAAATGCC
2468 ATCTAGTGAT GATGAGGCTA CTGCTGACTC TCAACATTCT ACTCCTCCAA AAAAGAAGAG AAAGGTAGAA
2538 GACCCCAAGG ACTTTCCTTC AGAATTGCTA AGTTTTTTGA GTCATGCTGT GTTTAGTAAT AGAACTCTTG
2608 CTTGCTTTGC TATTTACACC ACAAAGGAAA AAGCTGCACT GCTATACAAG AAAATTATGG AAAAATATTC
2678 TGTAACCTTT ATAAGTAGGC ATAACAGTTA TAATCATAAC ATACTGTTTT TTCTTACTCC ACACAGGCAT
2748 AGAGTGTCTG CTATTAATAA CTATGCTCAA AAATTGTGTA CCTTTAGCTT TTTAATTTGT AAAGGGGTTA
2818 ATAAGGAATA TTTGATGTAT AGTGCCTTGA TCATAATCAG CCATACCACA TTTGTAGAGG TTTTACTTGC
2888 TTTAAAAAAC CTCCCACACC TCCCCCTGAA CCTGAAACAT AAAATGAATG CAATTGTTGT TGTTAACTTG
2958 TTTATTGCAG CTTATAATGG TTACAAATAA AGCAATAGCA TCACAAATTT CACAAATAAA GCATTTTTTT

XbaI

3028 CACTGCATTC TAGTTGTGGT TTGTCCAAAC TCATCAATGT ATCTTATCAT GTCTGCCTCT AGAGCTGCAT
3098 TAATGAATCG GCCAACGCGC GGGGAGAGGC GGTTTGCGTA TTGGGCGCTC TTCCGCTTCC TCGCTCACTG
3168 ACTCGCTGCG CTCGGTCGTT CGGCTGCGGC GAGCGGTATC AGCTCACTCA AAGGCGGTAA TACGGTTATC
3238 CACAGAATCA GGGGATAACG CAGGAAAGAA CATGTGAGCA AAAGGCCAGC AAAAGGCCAG GAACCGTAAA
3308 AAGGCCGCGT TGCTGGCGTT TTTCCATAGG CTCCGCCCCC CTGACGAGCA TCACAAAAAT CGACGCTCAA
3378 GTCAGAGGTG GCGAAACCCG ACAGGACTAT AAAGATACCA GGCGTTTCCC CCTGGAAGCT CCCTCGTGCG
3448 CTCTCCTGTT CCGACCCTGC CGCTTACCGG ATACCTGTCC GCCTTTCTCC CTTCGGGAAG CGTGGCGCTT
3518 TCTCAATGCT CACGCTGTAG GTATCTCAGT TCGGTGTAGG TCGTTCGCTC CAAGCTGGGC TGTGTGCACG
3588 AACCCCCCGT TCAGCCCGAC CGCTGCGCCT TATCCGGTAA CTATCGTCTT GAGTCCAACC CGGTAAGACA
3658 CGACTTATCG CCACTGGCAG CAGCCACTGG TAACAGGATT AGCAGAGCGA GGTATGTAGG CGGTGCTACA
3728 GAGTTCTTGA AGTGGTGGCC TAACTACGGC TACACTAGAA GGACAGTATT TGGTATCTGC GCTCTGCTGA
3798 AGCCAGTTAC CTTCGGAAAA AGAGTTGGTA GCTCTTGATC CGGCAAACAA ACCACCGCTG GTAGCGGTGG

FIG. 12E

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3868 TTTTTTTGTT TGCAAGCAGC AGATTACGCG CAGAAAAAAA GGATCTCAAG AAGATCCTTT GATCTTTTCT
3938 ACGGGGTCTG ACGCTCAGTG GAACGAAAAC TCACGTTAAG GGATTTTGGT CATGAGATTA TCAAAAGGA
4008 TCTTCACCTA GATCCTTTTA AATTAAAAAT GAAGTTTTAA ATCAATCTAA AGTATATATG AGTAAACTTG
4078 GTCTGACAGT TACCAATGCT TAATCAGTGA GGCACCTATC TCAGCGATCT GTCTATTTCG TTCATCCATA
4148 GTTGCCTGAC TCCCCGTCGT GTAGATAACT ACGATACGGG AGGGCTTACC ATCTGGCCCC AGTGCTGCAA
4218 TGATACCGCG AGACCCACGC TCACCGGCTC CAGATTTATC AGCAATAAAC CAGCCAGCCG GAAGGGCCGA
4288 GCGCAGAAGT GGTCCTGCAA CTTTATCCGC CTCCATCCAG TCTATTAATT GTTGCCGGGA AGCTAGAGTA
4358 AGTAGTTCGC CAGTTAATAG TTTGCGCAAC GTTGTTGCCA TTGCTACAGG CATCGTGGTG TCACGCTCGT
4428 CGTTTGGTAT GGCTTCATTC AGCTCCGGTT CCCAACGATC AAGGCGAGTT ACATGATCCC CCATGTTGTG
4498 CAAAAAAGCG GTTAGCTCCT TCGGTCCTCC GATCGTTGTC AGAAGTAAGT TGGCCGCAGT GTTATCACTC
4568 ATGGTTATGG CAGCACTGCA TAATTCTCTT ACTGTCATGC CATCCGTAAG ATGCTTTTCT GTGACTGGTG
4638 AGTACTCAAC CAAGTCATTC TGAGAATAGT GTATGCGGCG ACCGAGTTGC TCTTGCCCGG CGTCAATACG
4708 GGATAATACC GCGCCACATA GCAGAACTTT AAAAGTGCTC ATCATTGGAA AACGTTCTTC GGGGCGAAAA
4778 CTCTCAAGGA TCTTACCGCT GTTGAGATCC AGTTCGATGT AACCCACTCG TGCACCCAAC TGATCTTCAG
4848 CATCTTTTAC TTTCACCAGC GTTTCTGGGT GAGCAAAAAC AGGAAGGCAA AATGCCGCAA AAAAGGGAAT
4918 AAGGGCGACA CGGAAATGTT GAATACTCAT ACTCTTCCTT TTTCAATATT ATTGAAGCAT TTATCAGGGT
4988 TATTGTCTCA TGAGCGGATA CATATTTGAA TGTATTTAGA AAAATAAACA AATAGGGGTT CCGCGCACAT
5058 TTCCCCGAAA AGTGCCACCT GACGTGACGT CTAAGAAACC ATTATTATCA TGACATTAAC CTATAAAAAT
5128 AGGGCGTATCA CGAGGCCCTT TCGTCTTCAA
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FIG. 12F

INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/US 96/10109

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/63 C12N15/67 C12N15/85 C12N15/62 C12N5/10

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	PROC NATL ACAD SCI U S A, JUL 3 1995, 92 (14) P6522-6, UNITED STATES, XP002015265 SHOCKETT P ET AL: "A modified tetracycline-regulated system provides autoregulatory, inducible gene expression in cultured cells and transgenic mice." see the whole document ---	1-20
P,X	WO,A,96 01313 (BUJARD HERMANN ;GOSSEN MANFRED (US)) 18 January 1996 see page 4-5; figure 9 ---	1-20
	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
7 October 1996	18. 10. 96

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Espen, J

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	PROC NATL ACAD SCI U S A, MAY 28 1996, 93 (11) P5185-90, UNITED STATES, XP002015266 HOFMANN A ET AL: "Rapid retroviral delivery of tetracycline-inducible genes in a single autoregulatory cassette [see comments]" see figure 1 ---	1-20
Y	DE,A,39 34 454 (MERCK PATENT GMBH) 18 April 1991 see page 6, line 27 - line 37; figures 3,4 ---	1-20
Y	PROC NATL ACAD SCI U S A, JUN 15 1992, 89 (12) P5547-51, UNITED STATES, XP000564458 GOSSEN M ET AL: "Tight control of gene expression in mammalian cells by tetracycline-responsive promoters." see the whole document -----	1-20

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inter ional Application No
PCT/US 96/10109

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9601313	18-01-96	AU-A- 3092395	25-01-96
DE-A-3934454	18-04-91	NONE	